Universidad Politécnica de Valencia
Departamento de Biotecnología

Abscisic acid signal transduction: Regulation by HAB1 and interaction with brassinosteroids mediated by BRX

Tesis Doctoral
Américo do Patrocínio Rodrigues

Director
Pedro Luis Rodríguez Egea

Universidad Politécnica de Valencia
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By
Américo do Patrocínio Rodrigues

Supervisor

Pedro Luis Rodríguez Egea

In a minute or two, the Caterpillar got down off the mushroom and crawled away into the grass, merely remarking as it went, "One side will make you grow taller, and the other side will make you grow shorter."
"One side of what? The other side of what?" thought Alice to herself.

Lewis Caroll, in *Alice's Adventures in Wonderland* (1865).

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Abstract

The phytohormone abscisic acid (ABA) is a key regulator of plant growth and development as well as of plant responses to stress namely water and salt stresses.

To gain further insight into ABA signaling and its role in growth regulation, we have screened for *Arabidopsis thaliana* mutants hypersensitive to ABA-mediated root growth inhibition. In this screen, we have identified a loss-of-function allele of *BREVIS RADIX (BRX*) in Columbia background, named *brx-2*. *BRX* encodes a key regulator of cell proliferation and elongation in the root, which has been implicated in the brassinosteroid (BR) pathway as well as regulation of auxin-responsive gene expression. Mutants affected in BR signaling that are not impaired in root growth, such as *bes1-D*, *bzr1-D* and *bsu1-D*, also showed enhanced sensitivity to ABA-mediated inhibition of root growth. Triple loss-of-function mutants affected in protein phosphatases type-2C (PP2Cs) that act as negative regulators of ABA signaling showed impaired root growth in the absence of exogenous ABA, indicating that disturbed regulation of ABA sensitivity impairs root growth. In agreement with this result, diminishing ABA-sensitivity of *brx-2* by crossing it with a *35S:HAB1* ABA-insensitive line allowed significantly higher recovery of root growth after BL treatment. Finally, transcriptomic analysis revealed that ABA treatment negatively affects auxin signaling in wild type and *brx-2* roots and that ABA response is globally altered in *brx*-2. Taken together, our results reveal an interaction between BRs, auxin and ABA in the control of root growth and indicate that fine tuning of ABA response might be required to prevent a deleterious effect on growth and development in the absence of environmental stress. ABA response is controlled by a complex network in which phosphorylation/dephosphorylation events have a very important role. HAB1 is a PP2C that plays a key role as negative regulator of ABA signaling; however, the molecular details of HAB1 action in this process are not known. A two hybrid screening revealed that AtSWI3B, an *Arabidopsis* homolog of the yeast SWI3 subunit of SWI/SNF chromatin remodeling complexes, is a prevalent interacting partner of HAB1. The interaction mapped to the N-terminal half of AtSWI3B and required an intact protein phosphatase catalytic domain. Bimolecular fluorescence complementation (BiFC) and coimmunoprecipitation (ChIP) assays confirmed the interaction of HAB1 and SWI3B in the nucleus of plant cells. Yeast two hybrid and BiFC assays also confirmed the interaction of SWI3B with ABI1, ABI2 and PP2CA, though this interaction was weaker than the observed with HAB1. *swi3b* mutants showed a reduced sensitivity to ABA-

mediated inhibition of seed germination and growth, and reduced expression of the ABA-responsive genes *RAB18* and *RD29B*. ChIP experiments showed that the presence of HAB1 in the vicinity of *RD29B* and *RAB18* promoters was abolished by ABA, which suggests a direct involvement of HAB1 in regulation of ABA-induced transcription. Contrary to the phenotypes presented by *swi3b* mutants, mutants of BRM and SYD, other putative members of Arabidopsis SWI/SNF complex, showed hypersensitivity to ABA in germination, root growth and enhanced expression of ABA responsive genes. Additionally, our results uncover AtSWI3B as a novel positive regulator of ABA signaling and suggest that HAB1 modulates ABA response through regulation of a putative SWI/SNF chromatin remodelling complex.

Resumen

El ácido abscísico (ABA) desempeña un papel decisivo en la regulación del crecimiento y desarrollo vegetal así como en la respuesta de la planta ante situaciones de estrés, en particular estreses hídrico y salino.

En un rastreo de mutantes hipersensibles al ABA, hemos identificado un nuevo alelo de pérdida de función del gen *BREVIS RADIX* (*BRX*), denominado *brx-2*. *BRX* codifica un regulador clave de la proliferación y elongación celular en raíz, implicado en la ruta de los brasinosteroides (BR) y en la regulación de la respuesta a auxinas. Mutantes afectados en la señalización por BRs, pero que no tienen afectado el desarrollo de la raíz, *bes1-D*, *bzr1-D* y *bsu1-D*, también muestran una mayor sensibilidad a ABA en estos ensayos. Por otra parte, mutantes triples de pérdida de función en las proteínas fosfatasa de tipo 2C (PP2Cs) que actúan como reguladores negativos de la ruta de ABA, muestran un fenotipo extremo de hipersensibilidad a ABA y un defecto en el crecimiento de raíz en ausencia de ABA exógeno. La aplicación post-embriónica de brasinólido (BL) sólo rescata parcialmente el defecto de crecimiento de raíz en mutantes *brx*; en cambio, al disminuir la sensibilidad a ABA de *brx-2* mediante cruce con una línea insensible a ABA (*35S:HAB1*), se consigue una mayor recuperación del crecimiento de raíz tras aplicar BL. El análisis transcriptomico muestra que el tratamiento con ABA afecta negativamente la señalización por auxinas en los mutantes *brx-2* y en las plantas silvestres. En conjunto, estos resultados muestran una interacción entre BRs y ABA en el control del crecimiento de raíz e indican que BRX modula la sensibilidad de la raíz al ABA. La regulación de la respuesta a ABA es controlada por diversos factores, de entre los cuales tienen un papel fundamental fenómenos de fosforilación/desfosforilación. HAB1 es una PP2C que, junto a otras PP2Cs del grupo A, desempeñan un papel clave como reguladores negativos de la ruta de señalización del ABA. Los sustratos de HAB1 no han sido identificados y por lo tanto se desconocen los detalles moleculares de su acción en la ruta hormonal. Mediante un rastreo de dos híbridos pudimos identificar AtSWI3B como una diana prevalente de interacción con HAB1. SWI3B es el homólogo en *Arabidopsis* de la subunidad SWI3B de levadura que forma parte de los complejos remodeladores de cromatina tipo SWI/SNF. La interacción está localizada en la mitad N-terminal de SWI3B, es específica para la fosfatasa HAB1 y requiere un dominio catalítico funcional en la PP2C, ya que la versión mutada *Gly246Asp hab1* no interacciona con AtSWI3B. Ensayos de interacción proteína-proteína de tipo BiFC y experimentos de co-

inmunoprecipitación confirmaron la interacción de HAB1 y AtSWI3B en el núcleo de células de tabaco, y interacción más débil con ABI1, ABI2 y PP2CA. Mutantes *swi3b* mostraban una sensibilidad reducida al ABA en ensayos de inhibición de germinación y crecimiento, así como una inducción muy atenuada de los genes *RAB18* y *RD29B* en ensayos de expresión génica. Experimentos de inmunoprecipitación de cromatina (ChIP) mostraban que la presencia de HAB1 en los promotores de *RD29B* y *RAB18* fue eliminada al añadir ABA exógeno, lo que sugiere una implicación directa de HAB1 en la regulación de la transcripción en respuesta a ABA. Al contrario del fenotipo de los mutantes *swi3b*, mutantes de BRM y SYD, otros miembros putativos de los complexos SWI/SNF de *Arabidopsis*, presentan fenotipos de hipersensibilidad a ABA en germinación y crecimiento de la raíz y un aumento de la expresión de genes de respuesta a ABA. Adicionalmente, estos resultados muestran que SWI3B es un nuevo regulador positivo de la señalización por ABA y sugieren que HAB1 modula la respuesta a la hormona mediante regulación de un complejo putativo de remodelación de cromatina del tipo SWI/SNF.

Resum

L'àcid abcísic (ABA) realitza un paper decisiu en la regulació del creixement y desenvolupament així com en la resposta de la planta front a situacions d'estrés, en particular estrés hídric y salí.

En un rastreig de mutantes hipersensibles a ABA, hem identificat un nou al.lel de pèrdua de funció del gen *BREVIS RADIX* (*BRX*), anomenat *brx-2*. *BRX* codifica un regulador clau de la proliferació i elongació cel·lular en arrel, implicat en la ruta dels brasinosteroids (BR) i en la regulació de la resposta a auxines. Mutants afectats en la senyalització per BRs, però que no tenen afectat el desenvolupament de l'arrel, *bes1-D*, *bzr1-D* i *bsu1-D*, també mostren una major sensibilitat a ABA en estos assajos. D'altra banda, mutants triples de pèrdua de funció en les proteïnes fosfatasa de tipus 2C (PP2Cs) que actuen com a reguladors negatius de la ruta d'ABA, mostren un fenotip extrem d'hipersensibilitat a ABA i un defecte en el creixement de l'arrel en absència d'ABA exogen. L'aplicació post-embriònica de brasinòlid (BL) només rescata parcialment el defecte de creixement d'arrel en mutants *brx*; en canvi, al disminuir la sensibilitat a ABA de *brx-2* mitjançant l'encreuament amb una línia insensible a ABA (*35S:HAB1*), s'aconseguix una major recuperació del creixement de l'arrel després d'aplicar BL. L'anàlisi transcriptòmic mostra que el tractament amb ABA afecta negativament la senyalització per auxines en els mutants *brx-2* i en les plantes silvestres. En conjunt, estos resultats mostren una interacció entre BRs i ABA en el control del creixement de l'arrel i indiquen que BRX modula la sensibilitat de l'arrel a l'ABA. La regulació de la resposta a ABA està controlada per diversos factors, d'entre els quals tenen un paper fonamental fenòmens de fosforilació/desfosforilació. HAB1 és una PP2C que, junt amb altres PP2Cs del grup A, exercixen un paper clau com a reguladors negatius de la ruta de senyalització de l'ABA. Els substrats de HAB1 no han sigut identificats i per tant es desconeixen els detalls moleculars de la seua acció en la ruta hormonal. Per mitjà d'un rastreig de dos híbrids vam poder identificar AtSWI3B com una diana prevalent d'interacció amb HAB1. SWI3B és l'homòleg en *Arabidopsis* de la subunitat SWI3B de rent que forma part dels complexos remodeladors de cromatina tipus SWI/SNF. La interacció està localitzada en la mitat N-terminal de SWI3B, és específica per a la fosfatasa HAB1 i requerix un domini catalític funcional en la PP2C, ja que la versió mutada Gly246Asp hab1 no interacciona amb AtSWI3B. Assajos d'interacció proteïna-proteïna de tipus BiFC i experiments de co-inmunoprecipitació van confirmar la interacció de HAB1 i AtSWI3B en el nucli de

cèl·lules de tabac, i una interacció més dèbil amb ABI1, ABI2 i PP2CA. Mutants *swi3b* mostraven una sensibilitat reduïda a l'ABA en assajos d'inhibició de germinació i creixement, així com una inducció molt atenuada dels gens *RAB18* i *RD29B* en assajos d'expressió gènica. Experiments d'inmunoprecipitació de cromatina (ChIP) mostraven que la presència de HAB1 en els promotors de *RD29B* i *RAB18* va ser eliminada a l'afegir ABA exogen, la qual cosa suggerix una implicació directa de HAB1 en la regulació de la transcripció en resposta a ABA. Al contrari del fenotip dels mutants *swi3b*, mutants de BRM i SYD, altres membres putatius dels complexos SWI/SNF d'Arabidopsis, presenten fenotips d'hipersensibilitat a ABA en germinació i creixement de l'arrel i un augment de l'expressió de gens de resposta a ABA. Addicionalment, estos resultats mostren que SWI3B és un nou regulador positiu de la senyalització per ABA i suggerixen que HAB1 modula la resposta a l'hormona per mitjà de la regulació d'un complex putatiu de remodelació de cromatina del tipus SWI/SNF.

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1. Introduction

1. Introduction

The term abscisic acid, and the acronym ABA, were proposed in 1968 (Addicott et al., 1968) as a compromise to designate the substance previously named by Addicott and co-workers abscisin II, an abscission accelerating substance isolated from cotton fruits, and dormin, by the group of Wareing, that was studying dormant buds in sycamore. In retrospect, it seemes that 'dormin' would have been a better name than 'abscisic acid' (Karssen, 2002).

ABA is a key regulator of plant growth and development as well as plant responses to stress, namely water and salt stresses. In addition to higher plants, ABA is also found in moss, fern, algae, fungi (Nambara and Marion-Poll, 2005) and animals (Le Page-Degivry et al., 1986; Zocchi e al., 2001; Scarfi et al., 2008).

Hormone response mutants have resulted of crucial importance to dissect the signal transduction pathways that control diverse physiological processes as well as genetic interactions among different signaling pathways (Gazzarrini and McCourt, 2001; Rock and Sun, 2005). Research in ABA signaling have resulted in the identification of many elements of the ABA signal transduction pathway, including both negative and positive regulators of the pathway (reviewed by Finkelstein et al., 2002; Himmelbach et al., 2003; Israelsson et al., 2006). Despite the large amount of information available, fundamental aspects of the role of ABA, its transduction pathway, and crosstalk with other hormones are still unclear.

As referred, ABA plays a key role in plant response to drought and salt stresses. Drought and salinity are the two major environmental factors that currently reduce plant productivity, being responsible, in severe conditions, for up to 65% reduction in yield, much higher than the losses caused by diseases and insects that, while devastating to individual farmers, globally are generally less than 10% (Serrano et al., 1999).

In a world with an increasing population, the possible reduction of water availability in some agricultural areas, due to climate change, and the reduction of crop yields and agricultural land, as a consequence of soil salinity, poses a challenging problem. To feed the increasing human population improvements in crop productivity are essential (Rosegrant and Cline, 2003). To achieve this objective, it is particularly important to better understand the mechanisms of plant adaptation to salt and water stresses and, therefore, the role ABA in these processes.

The role of abscisic acid in growth and development

The effect of ABA in growth and development depends on tissue, concentration and interaction with the environment.

The exogenous administration of ABA leads to inhibition of seed germination and, in general, to a reduction of root and shoot growth in seedlings. However, a growth-promoting role of endogenous ABA by controlling fertility and vegetative growth and determining organ and body size has also been described (see below). This apparent contradiction could be explained if ABA inhibits growth at high concentrations, but activates growth at low concentrations (Cheng et al., 2002; del Pozo et al., 2005).

The role of abscisic acid in the absence of stress

An essential function of endogenous ABA in the promotion of growth and development in the absence of stress is suggested by the growth phenotype of ABA-deficient mutants like *aba1* (Rock and Zeevaart, 1991; Finkelstein and Rock, 2002; Barrero et al., 2005) and *aba2* (Nambara et al., 1998; Cheng et al., 2002; Lin et al., 2007). The semi-dwarf phenotype of ABA-deficient mutants was sometimes attributed to their inability to retain water, as a result of impaired stomatal closure. However, ABA-deficient mutants grow less than wild type even in near-saturation humidity conditions, and addition of a small amount of exogenous ABA partially or completely restores the wild type phenotype (Barrero et al., 2005; Lin et al., 2007). This data suggest that basal levels of ABA are essential for maintaining normal plant growth.

In early stages of embryogenesis ABA may prevent seed abortion and promotes embryo growth (Cheng et al., 2002; Frey et al., 2004). The importance of ABA in early stages of embryogenesis is also suggested by its essential role for the acquisition of embryogenic competence by somatic cells (Senger et al., 2001; Kikuchi et al., 2006; Gaj et al., 2006). In contrast, during late embryogenesis ABA is responsible for induction and maintenance of seed dormancy (revised in Kucera et al., 2005; Kermode, 2005; Finch-Savage and Leubner-Metzger, 2006; Gutierrez et al., 2007; Finkelstein et al., 2008).

ABA is apparently involved in the control of root apical meristem (RAM) maintenance. Primary roots of *ABA INSENSITIVE 8*/*ELONGATION DEFECTIVE 1*, (*ABI8/ELD1*) mutants are impaired in elongation and cell differentiation. The root tip of

these mutants' seedlings had cells with meristematic characteristics but few or no cell divisions were found in the RAM, while meristematic cells continued transition to the elongation zone, resulting in meristem consumption (Cheng et al., 2002; Brocard-Gifford et al., 2004). Mutations in the *Arabidopsis* (*Arabidopsis thaliana*) TETRATRICOPEPTIDE-REPEAT THIOREDOXIN-LIKE 1 (TTL1) cause reduced tolerance to NaCl and osmotic stress that is characterized by reduced root elongation, disorganization of the root meristem, and impaired osmotic responses during germination and seedling development. Expression analyses of genes involved in ABA biosynthesis and catabolism suggest that TTL1 is not involved in the regulation of ABA levels but is required for ABA-regulated responses (Rosado et al., 2006). Another indication that ABA is involved in RAM maintenance is the fact that it rescues the RAM defects of the lateral root organ defective (*latd*) mutant of *Medicago truncatula* (Liang et al., 2007). These studies indicate that ABA can be involved in RAM maintenance and that transition from indeterminate to determinate growth may be ABA dependent (Shishkova et al., 2008). In *Arabidopsis* and tomato normal levels of ABA are also required to maintain shoot growth independently of effects on plant water balance (Sharp et al., 2000; LeNoble et al., 2004).

Another developmental process that that seems to be partially under control of ABA is flowering. In several species have been reported an inhibitory effect of ABA on floral initiation (e.g. Su et al., 2002; Wang et al., 2002a; Saniewski et al., 1999). However, in some plants ABA can promote flowering in short day photoperiod conditions (Podolnyi et al., 1989; Wilmowicz et al., 2008).

The role of abscisic acid under stress conditions

A critical function of ABA during vegetative growth is to optimize growth during environmental stress by maintaining osmotic homeostasis (Finkelstein and Rock, 2002). Drought, salt and osmotic stress activate a complex plant response, partially dependent of ABA, that involves different processes, namely control of stomatal aperture, accumulation of osmolites and control of root and shoot growth.

Plants lose much of their water through stomata. The opening and closing of stomata, and thus a considerable part of the plant's water management, is modulated by multiple factors such as CO_2 concentration, light, humidity, biotic stresses and

different phytohormones, namely ABA (Israelsson et al., 2006; Hirayama and Shinozaki, 2007; Nilson and Assmann, 2007; Acharya and Assmann, 2009). Under conditions of mild water stress as the soil starts to dry, and even before water potential of leaves is significantly affected, stomatal closure is induced, reducing water loss to cope with this stress situation. Traditionally, this process was explained by a release of root accumulated/synthesized ABA in the xylem vessels followed by its transport to shoots and leaves to regulate stomatal activity (reviewed in Ren et al., 2007; Jiang and Hartung, 2008). However, some reports indicate that in tomato and *Arabidopsis* shoot derived ABA is necessary and sufficient for stomatal closure (Holbrook, et al., 2002; Christmann et at., 2007) and suggest that: 1) other chemical or hydraulic signals may be involved in the root to shoot signaling (reviewed in Ren et al., 2007; Schachtman and Goodger, 2008); 2) most of the ABA biosynthesis induced by stress takes place in the aerial part of the plant. In support of this idea, in *Arabidopsis* the ABA biosynthetic enzymes 9-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3), the short-chain Alcohol Dehydrogenase ABA2, and ABSCISIC ALDEHYDE OXIDASE 3 (AAO3) are localized in vascular parenchyma cells (Endo et al., 2008). Interestingly, while the presence of ABA2 and AAO3 are detected in turgid and water-stressed leaves, NCED3 (enzyme that catalyses the first committed step toward ABA biosynthesis) and *NCED3* mRNA, are only detected is dehydrated leaves (Endo et al., 2008).

Salt salinity affects plants not only by a direct influence in the uptake of water by roots but also because high concentration of salts within the plant can be toxic (Munns and Tester, 2008). Vacuolar sequestration of Na^+ not only lowers Na^+ concentration in the cytoplasm but also contributes to osmotic adjustment to maintain water uptake (Zhu, 2003). ABA not only upregulates the tonoplast Na^+/H^+ antiporters AtNHX involved in the sequestration of Na^+ (Yokoi et al.,2002; Shi and Zhu, 2002; Cheong and Yun, 2007) but may also upregulates vacuolar H^+ pumps that generate the electrochemical gradient necessary for antiporters activity (Fukuda and Tanaka, 2006; Gaxiola et al., 2007). Accumulation of compatible solutes, most commonly sucrose, proline and glycine betaine, is an important mechanism in stress response not only balancing the osmotic pressure of ions sequestration in vacuole but also facilitating the uptake of water (Munns and Tester, 2008). The biosynthesis of proline is strongly induced by ABA through the upregulation of DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE (P5CS), which is a key enzyme in the synthesis of this amino acid (Verslues and Bray, 2006).

ABA plays also an important role in root and shoot response to water deficit. A crucial ABA-dependent adaptive feature that promotes survival of plants under water stress is the maintenance of root elongation, even in severe conditions, whereas shoot growth is rapidly inhibited (Spollen et al., 2000; van der Weele et al., 2000; Sharp, 2002; Munns and Tester, 2008). In maize the maintenance of primary root growth under water stress condition is dependent of a high ABA concentration, probably due to the inhibitory effect of ABA in the ethylene production (Spollen et al., 2000; Sharp, 2002; Sharp et al., 2004). The results obtained by Sharp, 2002 and 2004, show that under stress conditions a higher ABA content in the root tip promotes a higher root elongation rate, on the contrary of what occurs in well watered conditions in which higher ABA concentration causes a higher inhibitory effect. In tomato and *Arabidopsis*, normal levels of ABA are required to maintain shoot-growth independently of effects of hormone status on plant water balance (Sharp et al., 2000; LeNoble et al., 2004). Root growth is maintained by ABA under water deficit by different mechanisms that involve osmotic adjustment, cell wall loosening, promotion of antioxidant systems, regulation of ion transport and restriction of ethylene production (Ober and Sharp, 1994; Gaymard et al., 1998; Guan et al., 2000; Spollen et al., 2000; Sharp et al., 2004). Finally, it has been recently described a major QTL in maize that simultaneously affects ABA biosynthesis and root agronomical traits both under well-watered and water-stress conditions (Landi et al., 2007).

Lateral roots development, in contrast to primary root growth, under water stress conditions is inhibited and ABA is a critical component of this process (Deak and Malamy 2005; De Smet et al., 2006; Xiong et al., 2006; Nibau et al., 2008). This process could represent an adaptive response to drought stress conditions, since water is usually more available deep down in the soil and restriction of the horizontal proliferation of lateral roots in the topsoil and allocation of more resources to the growth of primary roots certainly would offer an advantage to the plants by expanding their domains of water supply (Xiong et al., 2006).

In contrast to its key role in the plant adaptive response to abiotic stress, ABA often affects disease resistance negatively by interfering at different levels with biotic stress signaling (Mauch-Mani and Mauch, 2005; Yasuda et al., 2008; Asselbergh et al., 2008) though in many cases can have a positive effect (reviewed in Asselbergh et al., 2008). *Arabidopsis* mutants either impaired in ABA biosynthesis (*aao3-2* and *aba2-12*)

or insensitive to ABA (*abi4*) show an increased susceptibility to *Pythium irregulare* and *Alternaria brassicicola* compared with the wild-type background, indicating that ABA is a positive signal involved in the activation of effective defenses against this pathogen, through at least two independent mechanisms: callose priming and regulation of defense gene expression through activation of jasmonic acid (JA) biosynthesis. Surprisingly, *aao3-2*, *aba2-12* and abi4 mutants are more resistant to *Botrytis cinerea* (Adie et al., 2007). The differential role of ABA within different plant–pathogen interactions suggests that ABA levels may be key to the fine-tuning of plant defenses against particular pathogens (Adie et al., 2007). Interestingly, all fungal ABA-producing strains isolated so far are associated with plants (Asselbergh et al., 2008) and a recent study demonstrates that *Pseudomonas syringae* type III-secreted effectors target the *Arabidopsis* ABA signaling pathway, namely inducing NCED3, a key enzyme of ABA biosynthetic pathway (de Torres-Zabala et al.,2007). These data suggest that elevation of plant ABA levels could function as a general pathogenic mechanism to suppress plant defenses (Asselbergh et al., 2008).

ABA signal transduction

Forward and reverse genetics, biochemical and cytological studies have allowed the identification of a large number of components involved in ABA signaling pathway, which globally constitute a complex web like network in which many elements directly or indirectly affect each other (figure 1). A list of mutations that change ABA response is presented in table 1.

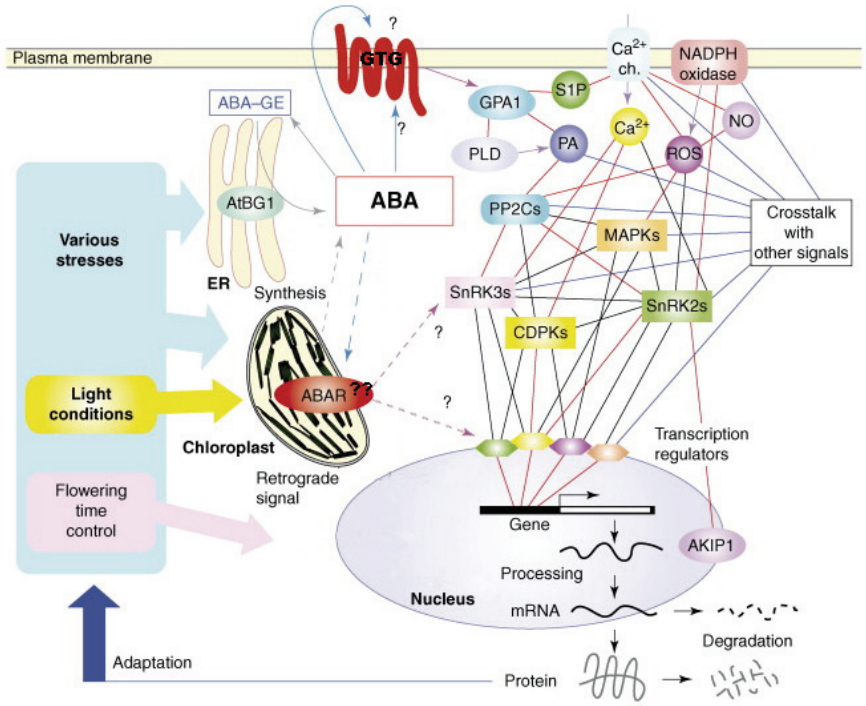

Figure 1 Simplified model of ABA signaling pathway. Abbreviations: Ca^{2+} ch., Ca^{2+} channel; CDPK, Ca^{2+}-dependent protein kinase; ER, endoplasmic reticulum; MAPK, mitogen-activated protein kinase; NO, nitric oxide; PA, phosphatidic acid; PP2C, protein phosphatase 2C; ROS, reactive oxygen species; SnRK, SNF1-related kinase; S1P, sphingosin-1-phosphate. (Based on Hirayama and Shinozaki, 2007)

Table 1. Mutations that change ABA response in *Arabidopsis thaliana*

[(a)] The indicated mutants are recessive except *abi1-1* and *abi2-1*. [(b)] The Insensitive or hypersensitive phenotype is indicated as I and H, respectively

Mutant [(a)]	**Phenotype** [(b)]	**Gene product**
abi1-1 (dominant)	I in seed and vegative tissues	PP2C Gly180Asp
abi1-2	H in seed, growth and stomatal closure	PP2C
abi1-1R1-7 intragenic revertant	H in seed and growth	PP2C Gly180Asp + second mutation
abi2-1 (dominant)	I in seed and vegative tissues	PP2C Gly168Asp
abi2-1R1 intragenic revertant	H in double mutant *abi1-1R4 abi2-1R1*	PP2C Gly168Asp + Glu186Lys
abi3	I in seed	TF B3 domain
abi4	I in seed	TF AP2 domain
abi5	I in seed	TF bZIP domain
abi8	I in seed and vegative tissues	Unknown function
ost1	I in stomatal closure	PK (SnRK2)
snrk2.2 snrk2.3 (double mutant)	I in seed germination and root growth inhibition	PK (SnRK2)
rcn1	I in seed and stomatal closure	PP2A regulatory subunit
rpk1	I in seed, growth, stomatal closure and gene expression	leucine-rich receptor-like kinase
gtg1gtg2 (double mutant)	I in seed, growth and stomatal closure.	GPCR-type G proteins
pldα1	I in stomatal closure and inhibition of stomatal opening	Phospholipase Dα1
rboh D/F (double mutant)	I in seed, growth and stomatal closure	NADPH oxidase (ROS produccion)
era1	H in seed and stomatal closure	Farnesil transferase
rop10	H in seed, growth and stomatal closure	Plasma membrane small GTPase.
pp2ac-2	H in seed and vegetative tissues	PP2A catalytic subunit
abh1	H in seed and stomatal closure	Nuclear cap-binding protein
fry1	*RD29A:LUC* H superinduction in seed	Inositol polyphosphate 1-phosphatase
sad1	*RD29A:LUC* Superinduction. H in seed and growth. Defectiv in ABA biosynthesis induction	Sm-like snRNP proteins (mRNA processing)
gcr1	H en growth, stomatal closure and gene expression	G protein coupled receptor
hyl1	H in seed and growth	Nuclear dsRNA binding protein involved in mRNA cleavage.
hab1	H in seed, growth and stomatal closure.	PP2C
pp2ca/ahg3	H in seed, growth and stomatal closure.	PP2C
ahg1	H in seed,	PP2C
gpa1	H in seed. I in inhibition of stomatal closure.	α subunit of a heterotrimeric GTP-binding protein

In the last years, four ABA-receptors have been described. In chronological order, FLOWERING TIME CONTROL PROTEIN A (FCA), ABA RECEPTOR/Mg-CHELATASE H SUBUNIT/GENOMES UNCOUPLED 5 (ABAR/CHLH/GUN5), G PROTEIN COUPLED RECEPTOR 2(GCR2) and two GPCR-type G proteins (GTG1 and GTG2) were reported as ABA receptors localized in nucleus, chloroplast, plasma membrane and also plasma membrane, respectively (Razem et al., 2006; Shen et al., 2006; Liu et al., 2007a; Pandey et al., 2009). However, a great controversy has surrounded this subject. Razem et al. (2006) article has been retracted (Razem et al., 2008). The role of GCR2 as an ABA receptor has been questioned since the *gcr2* and *gcl1* (GCR2 like1) single loss of function mutants as well as the double mutant showed similar ABA sensitivity than WT (Gao et al., 2007) and all double mutants, *gcr2 gcl1*, *gcr2 gcl2*, *gcl1 gcl2*, as well as the *gcr2 gcl1 gcl2* triple mutant displayed wild-type sensitivity to ABA in seed germination and early seedling development assays (Guo et al., 2008). It was also questioned the putative transmembrane structure of GCR2 (Johnston et al., 2007; Illingworth et al., 2008). Finally, whereas the *Arabidopsis* ABAR/CHLH/GUN5 specifically binds ABA and is stimulated by exogenous ABA (Shen et al., 2006), a recent report on the barley magnesium chelatase large subunit, XanF, very similar to AtCHLH with 82 % identical residues, showed that ABA had no effect on magnesium chelatase activity and did not bind to the barley XanF protein (Müller and Hansson, 2009). Moreover, barley magnesium chelatase mutants showed a wild-type response in respect to post-germination growth and stomatal aperture, questioning the validity of the idea that the large magnesium chelatase subunit is an abscisic acid receptor (Müller and Hansson, 2009).

Therefore, the only ABA receptors not questioned by the moment are GTG1 and GTG2. *gtg1-1 gtg2-1* double mutant presents a global ABA insensitive response (single T-DNA insertional mutants phenotype is similar to the WT one, suggesting that GTG1 and GTG2 might be functionally redundant) (Pandey et al., 2009). Interestingly, GTG1 and GTG2 interact with GPA1, a protein previously described as a partner of ABA signaling (Wang et al., 2001a; Pandey et al., 2006). The GTP-binding activity of the GTG1 and GTG2 proteins is accelerated by GPA1 and, remarkably, their GTPase activity is strongly inhibited (Pandey et al., 2009).

Finally, recent evidences from several laboratories indicate the existence of a new family of soluble ABA receptors localized at both nucleus and cytosol that inhibit clade A PP2Cs in an ABA dependent manner (Rodriguez, 2009; S. Cutler personal comunication).

ABA induces the production and accumulation in the cells of second messengers like Ca^{2+}, phosphatidic acid (PA) or reactive oxygen species (ROS), which in turn play an important role in the regulation of different components of ABA signal transduction like for instance protein kinases and protein phosphatases.

Protein phosphorylation/dephosphorylation events in ABA signaling involve several known protein kinases (PK) and phosphatases (Leung and Giraudat, 1998; Finkelstein et al., 2002). For instance, the guard cell-specific ABA-activated serine-threonine PK (AAPK) from *Vicia fava* and the orthologous OPEN STOMATA 1/SNF1-RELATED PROTEIN KINASE 2.6 (OST1/SnRK2.6) regulate ABA-induced stomatal closure (Li et al., 2000; Mustilli et al., 2002). Two other SnRK2, SnRK2.2 and SnRK2.3, regulate ABA response in germination, growth and gene expression (Fujii et al., 2007). Another PK involved in ABA signaling is PKABA1, which is induced by ABA and suppresses gibberellin (GA) inducible gene expression in barley aleurone layers (Gomez-Cadenas et al., 1999). In addition to the above mentioned calcium-independent PK, there are several calcium dependent PK implicated in ABA signaling that belong either to the CDPK or the SnRK3 family. The constitutively active expression of CDPK1 and CDPK1a activate ABA dependent promoters (Sheen, 1996). Recently, a reverse genetic analysis has uncovered a critical role for two CDPKs, CDPK3 and CDPK6, in regulating guard-cell aperture during responses to environmental stimuli (Mori et al., 2006). Two other CDPKs, CPK4 and CPK11, positively regulate ABA signal transduction in seed germination, seedling growth and stomatal movement through a mechanism that likely involves the phosphorilation of ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR 1 (ABF1) and ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR 4 (ABF4) (Zhu, et al., 2007). With respect to SnRK3s/CIPKs (CBL-interacting PK) several members of this family, PKS3, PSK18, CIPK3 and CIPK23 are involved in the regulation of ABA signaling (Guo et al., 2002; Gong et al., 2004; Cheong et al., 2007). Finally, several reports confirm the involvement of mitogen-activated protein kinases (MAPKs) in ABA dependent response to different stresses (Boudsocq and Laurière, 2005; Zhang et al., 2006) and germination (Lu et al., 2002).

The role of protein phosphates as counterpartners of protein kinases in phosphorylation/dephosphorylation processes will be referred latter.

Many ABA-inducible genes contain a conserved *cis*-acting element named ABRE (ABA-responsive element) (Yamaguchi-Shinosaki and Shinozaki, 2006). Optimal

ABA responsiveness usually requires a second *cis*-element or coupling element (CE), most being similar to ABREs (Nakashima et al., 2009). In *Arabidopsis*, the dehydration responsive element/C-Repeat (DRE/CRT) sequence may serve as a coupling element of ABRE in response to ABA (Narusaka et al., 2003; Sakuma et al., 2006; Nakashima et al., 2006), suggesting the existence of the interaction between the drought responsive element binding protein (DREB) regulons and the ABRE-related regulons (Nakashima et al., 2009). ABRE-binding (AREB) proteins / ABRE-binding factors (ABF) bind to ABRE and activate the expression of ABA response genes. Most of the ABFs are mainly expressed in vegetative tissues, whereas other bZIP transcription factors (TF) like ABI5 are active mainly during seed germination and early seedling development (Himmelbach et al., 2003; Yamaguchi-Shinosaki and Shinozaki, 2006). Some TFs that function as transcriptional repressors of ABA response have been also described (Himmelbach et al., 2002; Song et al., 2005; Pandey et al., 2005).

ABA response is also affected by post-transcriptional processes. The disruption of *ABH1*, *CBP80* and *CBP20* genes, enconding mRNA cap-biding proteins, leads to altered sensitivity of plants to ABA and stress (Hugouvieux et al, 2001; Xiong et al., 2001; Papp et al., 2004) and AHG2 is hypothesized to be necessary for mRNA poly(A) tail processing (Nishimura et al., 2005). In addition, control of RNA stability by micro-RNAs seems to play an important role in ABA signaling (Wasilewska et al., 2008).

Finally, the deep impact of ABA on the regulation of gene expression and the large number of TF involved may reflect a role of chromatin remodeling in ABA response, as suggested by some reports of chromatin modifying factors that affect ABA response (e.g. Song et al., 2005; Sridha and Wu, 2006), which will be referred later.

Clade A PP2Cs are negative regulators of ABA Signaling

Protein phosphorylation/dephosphorylation is a common and versatile regulatory mechanism that plays a key role in the control of many cellular processes. There are over 1000 *Arabidopsis* genes that code for protein kinases (Wang et al., 2003) and 150 genes that encode protein phosphatases (Kerk et al., 2008).

The PP2C class includes a group of serine threonine phosphatases that are dependent on Mg^{2+} or Mn^{2+} for their activity, are active as monomers and insensitive to known phosphatase inhibitors (Rodriguez, 1998; Cohen, 2004). Comprising 76 genes, PP2Cs represents the larger class of *Arabidopsis* protein phosphatases. The analysis

of public available genome data from other higher plants like poplar or rice shows that they contain a similar number of putative PP2Cs. The surprising high number of PP2Cs found in plants compared to other organisms (in humans only 16 PP2C genes have been identified (Lammers and Lavi, 2007)), might suggest a broader functional diversity than in other eucaryotes (Schweighofer et al., 2004).

The 76 *Arabidopsis* PP2C genes fall into ten groups except for 6 genes that could not be clustered (Schweighofer et al., 2004). Clade A comprises a set of PP2Cs with an important role in ABA signaling (figure 2).

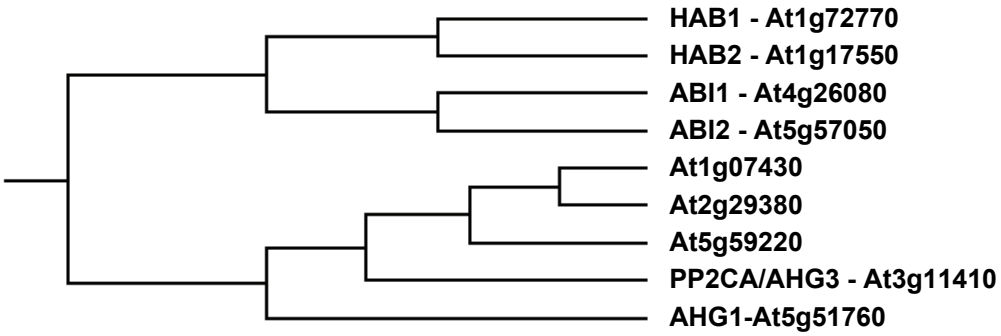

Figure 2. Clade A PP2Cs. Topographic cladogram based on amino acid sequence alignment of *Arabidopsis* clade A type-2C protein phosphatases.

Clade A PP2Cs present a very similar catalytic core, in size and amino acid sequence, with 11 characteristic conserved motivs of PP2C (Rodriguez, 1998; Schweighofer et al., 2004). The N-terminal domain presents low sequence similarity and a variable size suggesting that N-terminal part of the proteins possibly mediates the interactions with cellular substrates, regulatory proteins or secondary messengers (Rodriguez, 1998; Meinhard et al., 2002). The same conclusion is suggested by more recent data, for instance that: the phosphatidic acid (PA) binding region in ABA INSENSITIVE1 (ABI1) is located at its N terminus and a specific mutation in the N-terminal region (R73A) abolishes ABI1 binding to PA and blocks PA-conferred inhibition of ABI1 PP2C activity (Zhang et al., 2004; Mishra et al., 2006); and the N-terminal fragment of ABI1 comprising of amino acids 1–93 is as efficient as the entire protein in docking to MAP KINASE 6 (MPK6) by both the criteria of *in vitro* and yeast two-hybrid tests (Leung et al., 2006).

The pioneering evidence for the involvement of PP2Cs in ABA-signaling was provided by the identification of the ABA-insensitive *abi1-1* and *abi2-1* mutants and the

cloning of the corresponding loci (Koornneef et al., 1984; Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997; Rodriguez, et al., 1998). Currently, at least six *Arabidopsis* PP2Cs, namely ABI1, ABI2, PP2CA/AHG3, AHG1, HYPERSENSITIVE TO ABA1 (HAB1) and HYPERSENSITIVE TO ABA2 (HAB2), are known to regulate ABA signaling. Evidence obtained through genetic approaches indicates that these PP2Cs act as negative regulators of ABA signaling (Gosti et al., 1999; Merlot et al., 2001; Tahtiharju and Palva, 2001; Gonzalez-Garcia et al., 2003; Saez et al., 2004; Leonhardt et al., 2004; Yoshida et al., 2006b; Kuhn, et al., 2006; Saez et al., 2006; Nishimura et al., 2007). AHG1 and PP2CA/AHG3 appear to play an essential role for ABA signaling in germination and post-germination growth (Kuhn, et al., 2006; Yoshida et al., 2006b; Nishimura et al., 2007), but the *ahg1-1* mutant has no ABA-related phenotype in adult plants (Nishimura et al., 2007). The analysis of a *ahg1-1 ahg3-1* double mutant suggests that AHG1 has specific functions in seed development and germination, shared partially with AHG3 (Nishimura et al., 2007). On the other hand, the work of Kuhn, et al., (2006) suggests that PP2CA/AHG3 plays an essential role for ABA signaling both in seed and vegetative tissue, as the *pp2ca-1* mutant showed ABA-hypersensitivity in germination, growth and stomatal closure assays. Conversely, 35S:PP2CA expression caused ABA insensitivity in seed germination and ABA-induced stomatal closure assays. Previous work of Sheen (1998) showed that PP2CA can block ABA-induced gene induction when transiently over-expressed in protoplasts.

Single reduction/loss-of-function alleles from *ABI1*, *ABI2* and *HAB1* produced phenotypic effects on ABA signaling to different extent and it was apparent from double mutant analyses that some functional redundancy occurs among them (Merlot et al., 2001; Saez et al., 2006). For instance, inactivation of both *HAB1* and *ABI1* led to a stronger response to ABA than that found in either *hab1-1* or *abi1-2* monogenic mutants. A similar trend was obtained by combination of the recessive *abi1-1R4* and *abi2-1R1* alleles (Merlot et al., 2001).

Combined inactivation of close members of a gene family is usually required to unravel possible functional genetic redundancy and to establish a functional hierarchy among them. This fact has been particularly evident in hormonal signaling pathways (Hua and Meyerowitz, 1998; Higuchi et al., 2004; Achard et al., 2006; Iuchi et al., 2007). Alternatively, gain-of-function approaches can circumvent genetic redundancy, as deduced from the global ABA-insensitive phenotype found in the dominant mutants *abi1-1D* and *abi2-1D* as well as the transgenic lines 35S:HAB1, 35S:PP2CA and

35S:*hab1Gly246Asp* (Koornneef et al., 1984; Saez et al., 2004; Kuhn et al., 2006; Robert et al., 2006).

Triple mutants, *hab1-1 abi1-2 abi2-2* and *hab1-1 abi1-2 pp2ca-1* show an extreme response to exogenous ABA, impaired growth and constitutive response to endogenous ABA (Rubio et al., 2009) indicating that endogenous ABA levels are enough to trigger a constitutive response to ABA in the absence of PP2C negative regulators. Comparison of ABA response in the different mutants reveals that a progressive increase in ABA-sensitivity can be obtained through combined inactivation of these PP2Cs suggesting that ABA response is finely tuned by the integrated action of these genes, which is required to prevent a constitutive response to endogenous ABA that might have a deleterious effect in growth and development in the absence of environmental stress (Rubio et al., 2009).

The knowledge about PP2C targets in plants is scarce and scattered. Both ABI1 and ABI2 interact with *Arabidopsis GLUTATHIONE PEROXIDASE3* a protein that plays an important role in the regulation of plant water transpiration (Miao et al., 2006) and with the SOS2-like kinase PKS3/SnRK3.1 (Guo et al., 2002). ABI1 interacts with OST1/SnRK2.6 a kinase that plays a crucial role in the control of stomata (Yoshida et al., 2006a) and the homeodomain protein ATHB6, belonging to the plant-specific HD-Zip class (Himmelbach et al., 2002). Targets of ABI2 include three SnRK3, SnRK3.11/SOS2, SnRK3.13/PKS11 and SnRK3.15/PKS24 (Ohta et al., 2003) and fibrillin a lipid-binding proteins of plastids that are induced under abiotic stress conditions (Yang et al., 2006). Interestingly, PP2CA interacts with the potassium channel AKT2 (Cherel et al., 2002) and At1g07430 another PP2C from the same branch, interact with the potassium channel AKT1 (Lee et al., 2007).

ABA and hormonal crosstalk

The first ABA-deficient mutants of *Arabidopsis* were originally identified by screening for suppressor mutations of the germination defect of *ga1* mutant (Koornneef et al., 1982). By contrast, genetic screens for suppressors of the dominant ABA-insensitive mutation abi*1-1* identified some mutants that are defective in GA synthesis or response (Steber et al., 1998). *era3* mutants, which were originally identified as ABA hypersensitive, were found to be allelic to *ein2* (Ghassemian et al., 2000). Furthermore, *ctr1* and *ein2* mutants were identified as enhancers and suppressors of

abi1 mutants, respectively (Beaudoin et al., 2000). These and other similar findings clearly show that developmental processes are regulated by the interaction of multiple hormones. However, it is still not clear how crosstalking among hormones occurs at the molecular level.

Brassinosteroids (BR) are generally considered antagonists of ABA (e.g. Friedrichsen et al., 2002; Nemhauser and Chory, 2003; Bajguz, 2009). However, good candidates that might explain at the molecular level the ABA-BR crosstalk have not been identified.

BR is perceived by BRASSINOSTEROID INSENSITIVE1/ BRI1 KINASE INHIBITOR1 (BRI1/BKI1) receptor complex at the plasma membrane causing the release of BKI1 (He et al., 2000; Wang et al., 2001a; Kinoshita et al., 2005; Wang and Chory, 2006). The subsequent dimerization of BRI1 and BRI1 ASSOCIATED RECEPTOR KINASE1 (BAK1) activates a downstream signal transduction pathway that leads to BRI1 EMS SUPPRESSOR1 (BES1) and BRASSINAZOLE RESISTANT1 (BZR1) dephosphorylation (Li et al., 2002; Wang et al., 2002b; Yin et al., 2002; Li and Jin, 2007). BES1 and BZR1 activities are regulated by a GSK3-like kinase BIN2 (Choe et al., 2002; Pérez-Pérez et al., 2002). BIN2 phosphorylates BES1 and BZR1 and negatively regulates their function (Vert and Chory, 2006; Ryu et al., 2007). BR signaling through BRI1 inhibits BIN2 function by an unknown mechanism, leading to accumulation of non-phosphorylated BES1/BZR1 in the nucleus. The dephosphorylation of BES1 is facilitated by BSU1 phosphatase, which is required for accumulation of non-phosphorylated BES1 (Mora-Garcia et al., 2004). The non-phosphorylated forms of BES1 and BZR1 are capable of binding promoter elements in BR-regulated genes (Ryu et al.,2007).

ABA induces dormancy during embryo maturation (reviewed in Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al, 2002; Finkelstein et al., 2008; Gutierrez et al., 2007; Kermode, 2005), can inhibit germination and its accumulation correlates with the onset of dormancy. Dormancy is reduced in seeds that are ABA-deficient owing to mutations, chemical inhibition of biosynthesis, or sequestering of ABA by antibodies expressed in seeds, whereas overexpression of ABA biosynthetic enzymes leads to enhanced dormancy (Finkelstein et al., 2008). Genetic studies have shown that ABA produced by maternal tissues or supplied exogenously is not sufficient to induce dormancy implying that this is a form of embryo-controlled dormancy dependent

on ABA synthesis in the embryo and/or endosperm (Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al, 2002; Finkelstein et al., 2008).

Gibberellins counteract the effect of ABA playing an important role in dormancy release and in the promotion of germination. The non-germinating phenotype of GA-deficient mutants can be overcome by an additional mutation in an ABA-deficient locus and similarly ABA-deficient mutants can germinate in the presence of inhibitors of GA biosynthesis such as paclobutrazol (Seo et al., 2006). More than the absolute concentration of ABA and GA it seems that is the ABA/GA ratio the key regulator of both maturation and germination processes (Santos-Mendoza et al,,2008).

BR biosynthesis (*det2-1*) and signaling (*bri1-1*) mutants are ABA hypersensitive in germination suggesting that BR signal is needed to overcome inhibition of germination by ABA (Steber and McCourt, 2001). In *Arabidopsis* BR rescues the germination phenotype of severe GA biosynthetic mutants and of the GA-insensitive mutant *sleepy1*, maybe by stimulating GA biosynthesis (Steber and McCourt, 2001) but in tobacco seed germination is stimulated by BR and GA by different mechanisms (Leubner-Metzger, 2001).

The BR biosynthetic mutants, *sax1*, was identified based on its hypersensitivity to ABA and auxin in root growth (Ephritikhine et al., 1999). BR signal transduction mutants such as *bri1*, *bin2/dwf12* and *bin5* also display hypersensitivity to ABA inhibition of primary root growth (Clouse et al., 1996; Li et al.,2001; Choe et al.,2002). On contrary, roots of plants overexpressing the positive regulator BRASSINOSTEROID ENHANCED EXPRESSION 1 (BEE1) are more responsive to exogenous BL and partially insensitive to ABA (Friedrichsen et al.,2002). Interestingly, in WT plants ABA represses the expression of BEE1, BEE2 and BEE3 (Friedrichsen et al. 2002).

The antagonism between BR and ABA is also suggested by the enhanced stomatal closure of the BR-deficient *sax1* mutant (Ephritikhine et al., 1999). However, in *Vicia faba*, brassinolide, a specific BR, modulated stomatal aperture by promoting stomatal closure and inhibiting stomatal opening (Haubrick et al., 2006). These results suggest that interaction between BR, ABA and guard cell output may occur in a species-specific manner (Haubrick et al., 2006). Under heat treatment, crosstalk between ABA and BR is also suggested by the increase of ABA endogenous concentration as result of BR treatment in *Brassica napus* (Kurepin et al., 2008) and *Chlorella vulgaris* (Bajguz, 2008).

Chromatin and ABA signaling

The nucleosome is the elemental repeating unit in all eukaryotic chromatin and consists of 2 copies each of the 4 core histones H2A, H2B, H3, and H4. One tetramer of (H3/H4)2 and 2 dimers of (H2A/H2B) form the histone octamer, around which 147 bp of DNA are wrapped in 1.7 turns of a tight superhelix (Luger et al., 1997). The packaging of DNA into chromatin imposes profound and ubiquitous effects on almost all DNA-related metabolic processes including transcription, recombination, DNA repair, replication, kinetochore and centromere formation (Li et al., 2007). Also nucleosomes are themselves stable and show limited mobility they present dynamic proprieties due to the action of nucleosome–modifying complexes. Two general classes of chromatin-modifying factors can be distinguished, those that covalently modify the N-terminal tails of histone proteins and those that utilize ATP hydrolysis to remodel or reposition nucleosomes (Carrozza et al., 2003; Smith and Peterson, 2005).

Modifying complexes add or remove covalent modifications at particular residues on the histone proteins, that are recognized by transcriptional regulators and other factors (Saha et al., 2006). Some of these modifications have been correlated with transcriptional activation: acetylation, methylation, phosphorylation, and ubiquitination, whereas other: methylation, ubiquitination, sumoylation, deimination, and proline isomerization have been implicated in repression (Kouzarides, 2007). However the truth is likely to be that any given modification has the potential to activate or repress under different conditions (Kouzarides, 2007). Protein complexes use ATP hydrolysis to alter the histone-DNA contacts, including transient unwrapping of the end DNA from histone octamers, forming the DNA loop, or moving nucleosomes to different translational positions (sliding), all of which modify the accessibility of nucleosomal DNA to transcription factors (Li et al., 2007).

Covalent histone modifications and ABA signaling

In plants, histone modification regulate gene expression in response to diverse exogenous stimuli including biotic and abiotic stress and also play a central role in developmental regulation (Pfluger and Wagner, 2007). There is increasing evidence indicating that histone modifications are involved in plant response to ABA and to abiotic and biotic stresses.

During seed development, abscisic acid plays an important role in storage reserve accumulation and acquisition of desiccation tolerance. The onset of intense seed storage protein accumulation requires the presence of a B3 domain transcription factor such as ALF (in bean), ABI3 (in *Arabidopsis*), or VP1 (in *Zea mays*). Phaseolin gene (*phas*, enconding a major seed storage protein) is repressed in vegetative tissues but its exprestion is activated in seeds by chromatin modifications. Repression of *phas* expression is related to the deacetylated chromatin structure of phas promoter in vegetative tissues. In seeds, ABA induced expression of PvALF is required for *phas* expression (Li et al., 1999). PvALF mediate acetylation of H3-K9 and H4-K12 through histone acetyltransferases (HAT) whereas ABA induces acetylation of H3-K14 and methylation of H3-K4 (Ng et al., 2006). These histone modifications may recruit a chromatin-remodeling complex such as SWI/SNF, resulting in a decrease in histone–DNA interactions (Ng et al., 2006).

HISTONE MONOUBIQUITINATION1 (HUB1) and HUB2 are both required for monoubiquitination of H2B. HUB1 and HUB2 mutants show some developmental defects including reduced seed dormancy, and a much lower expression of some dormancy related genes, like DELAY OF GERMINATION 1 (DOG1), NCED9 and CYP707A2 has been detected in freshly harvested seeds of *hub1-2* (Liu et al., 2007b). These results suggest that HUB1 influence the expression of downstream genes associated with seed dormancy possibly through the monoubiquitination of H2B (Liu et al., 2007b).

Genetic analysis identified AtERF7, a member of the ethylene-responsive element binding factor family, as an important transcriptional repressor in ABA responses. AtERF7 interacts with PKS3 and can be phosphorylated by PKS3 in vitro. AtERF7 also interacts with the transcriptional corepressor, AtSin3, which in turn may interact with the histone deacetylase HDA19. AtSin3 and HDA19 enhance the transcription repression activity of AtERF7 (Song et al., 2005). Overexpression of AtERF7 in transgenic *Arabidopsis* plants reduced ABA responses in guard cells and decreased drought tolerance, whereas reductions in AtERF7 expression caused ABA hypersensitivity in guard cells, seed germination, and seedling growth (Song et al., 2005).

The expression of *Arabidopsis* HD2-type histone deacetylases (HDAC), AtHD2C, can be repressed by ABA and 35S:AtHD2C-GFP transgenic plants are insensitive to ABA, sodium chloride and mannitol inhibited root growth. In addition, 35S:AtHD2C-GFP transgenic plants displayed more tolerance to salt and drought

stresses compared with the wild-type and present up-regulation of *RD29B* and *RAB18* genes (Sridha and Wu, 2006).

In rice, the histone deacetylases HDA702 and HDA704 are induced by ABA, alicylic acid (SA) and jasmonic acid (JA), but surprisingly while expression of HDA702 is increased by cold, mannitol or NaCl treatments the expression of HDA704 is decreased by these treatments (Fu et al., 2007).

The *Arabidopsis HIGH EXPRESSION OF OSMOTIC STRESS RESPONSIVE GENES15* (*hos15*) mutant are hypersensitive to ABA and NaCl in terms of germination, and are hypersensitive to freezing stress. HOS15 can interact with histone H4 and the *hos15* mutant accumulates more acetylated histone H4 than WT plants. Moreover, in hos15 mutant plants the promoter of the RD29A gene is more active and is associated with histone H4 that is more acetylated, suggesting that RD29A, and likely other genes involved in stress tolerance, are repressed through a function of HOS15 in histone deacetylation (Zhu et al, 2008).

In Tobacco BY-2 cells and *Arabidopsis* T87 cells ABA, high salinity and cold stress trigger rapid and transient upregulation of histone H3 Ser-10 phosphorylation, H3 phosphoacetylation, and H4 acetylation followed by stress-type-specific gene expression (Sokol et al., 2007). In *Arabidopsis*, in response to drought stress H3K4me3 and H3K9ac are enriched on the coding regions of RESPONSIVE TO DESSICATION RD29A, RD29B, RD20 and RELATED TO AP2.4 (RAP2.4) this enrichment being consistent with transcriptional activation (Kim et al., 2008).

Role of ATP dependent chromatin remodeling complexes in ABA and stress responses

The catalytic subunits of chromatin remodeling complexes (CRC) are members of the SNF2 (sucrose nonfermenting 2) family of DNA-dependent ATPases (Kwon and Wagner, 2007). The genome of *Arabidopsis* encodes 41 of these proteins (Figure 3). Homology searches identify BRAHMA (BRM), At2g46020, SPLAYED (SYD), At2g28290, CHR12, At3g06010, and CHR23, At5g19310, as the closest homologues of yeast and animal SWI/SNF ATPase subunits (Knizewski et al., 2008)

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The role of these CRC in plants remains poorly understood. However a few reports connecting CRC with ABA and stress responses have been published.

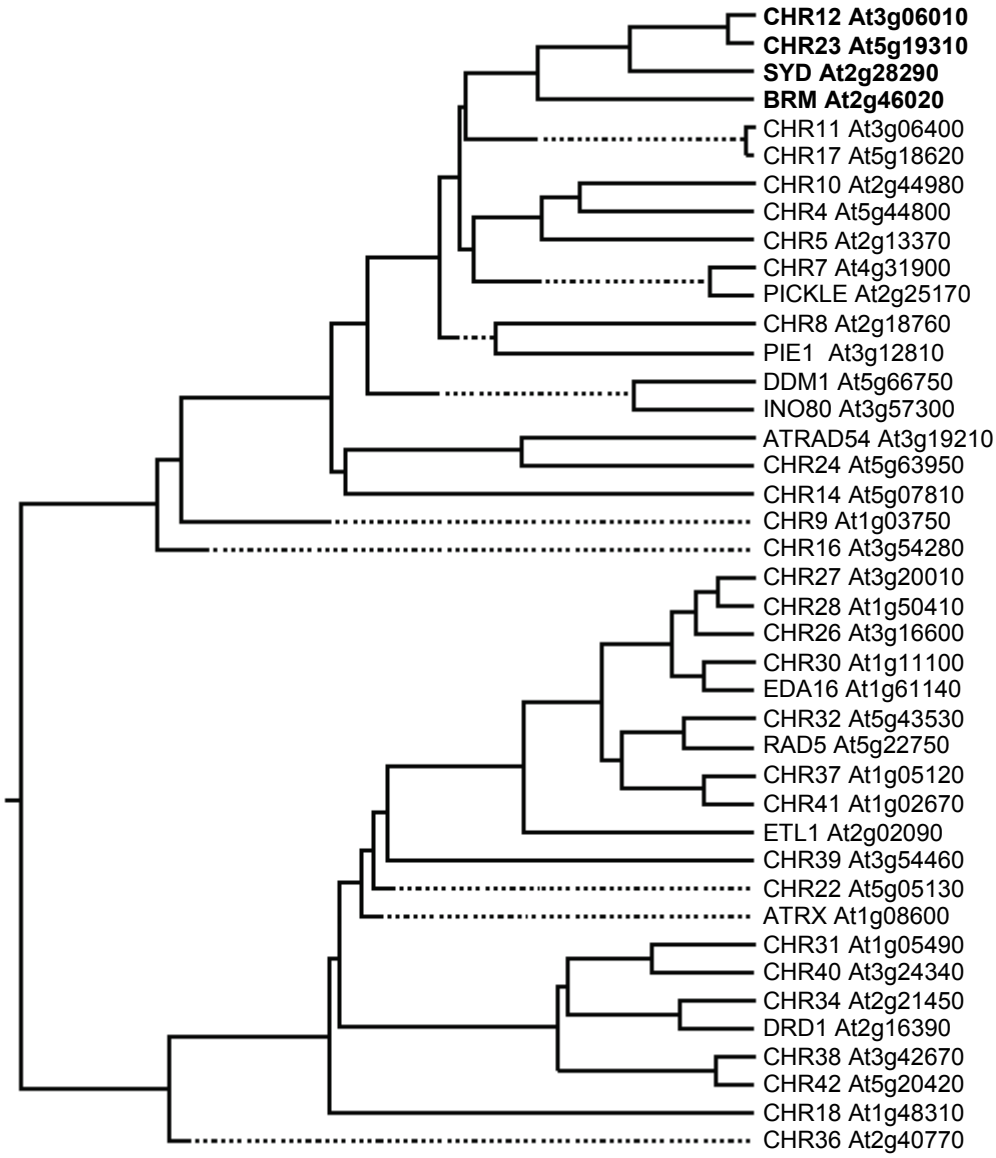

Figure 3. Cladogram based on amino acid sequence alignment of *Arabidopsis* Snf2 family ATPases. In bold BRM, SYD, CHR12, and CHR23, the *Arabidopsis* closest homologues of yeast and animal SWI/SNF ATPase subunits.

PICKLE (PKL) is necessary to repress *ABI3* and *ABI5* expression in response to ABA during germination. *pkl* mutants over express *ABI3* and *ABI5* in response to ABA and *ABI5* over expression leads to a hypersensitive germination response (Perruc et al., 2007).

*Ps*SNF5, a SNF5-like gene from pea with high similarity with *Arabidopsis* BSH, is transcribed during seed maturation and its mRNA levels are maintained during seed imbibition, but is induced by ABA and hydric stress in both seedlings and adult tissues and in a lesser extent in embryos, suggesting that SWI/SNF chromatin remodeling may play a role in the transmission of ABA signal and, therefore, in drought protection. (Rios et al., 2007).

Under non stress conditions *Arabidopsis* CHR12, a SWI/SNF ATPase, mutant plants are indistinguishable from the wild-type. Exposing CHR12 over expressing mutants to stress conditions leads to growth arrest of normally active primary buds, as well as to reduced growth of the primary stem. In contrast, the AtCHR12 knockout mutant shows less growth arrest than the wild-type when exposed to moderate stress. Modulation of AtCHR12 expression correlates with changes in expression of dormancy-associated genes. (Mlynarova et al., 2007).

Another SWI/SNF ATPase, SPLAYED (SYD), regulates specific stress signaling pathway being required for resistance against the necrotrophic pathogen *Botrytis cinerea* but not the biotrophic pathogen *Pseudomonas syrinis*. SYD is required for the expression of selected genes downstream of the jasmonate and ethylene signaling pathways (Walley et al., 2008).

Some of these data indirectly suggest that SWI/SNF-type complexes might be involved in ABA signaling and response to stress. All prototype SWI/SNF-type complexes studied so far contain a minimal structural and functional core composed of four types of subunits: SWI2/SNF2 (the ATPase, the major catalytic subunit), SNF5, SWI3 and SWP73. Until now, no complete plant CRC has been isolated and characterized. However, experimental data and database surveys indicate that the existence of SWI/SNF-type complexes in plants is highly probable with the gamut of possibilities shown in figure 3 (Jerzmanowski, 2007).

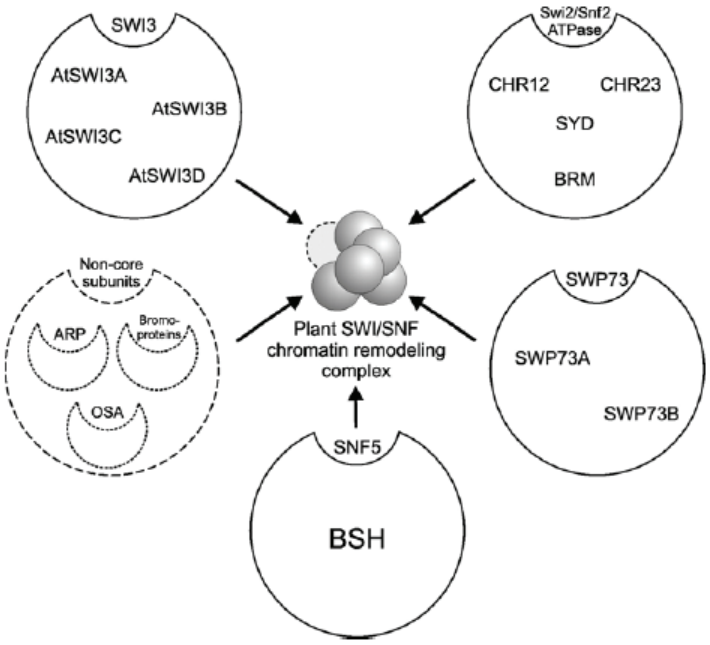

Figure 3. The *Arabidopsis* SWI/SNF complexes that can potentially be assembled from combinations of identified core subunits (in solid circles) and unknown candidate auxiliary sub-units (broken-line circle) (Jerzmanowski, 2007).

2. The short-rooted phenotype of the *brevis radix* mutant partly reflects root ABA hypersensitivity

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(*)These authors contributed equally to this work.

Abstract

To gain further insight into ABA signaling and its role in growth regulation, we have screened for *Arabidopsis thaliana* mutants hypersensitive to ABA-mediated root growth inhibition. As a result, we have identified a loss-of-function allele of *BREVIS RADIX (BRX*) in Columbia background, named *brx-2*, which shows enhanced response to ABA-mediated inhibition of root growth. *BRX* encodes a key regulator of cell proliferation and elongation in the root, which has been implicated in the brassinosteroid (BR) pathway as well as regulation of auxin-responsive gene expression. Mutants affected in BR signaling that are not impaired in root growth, such as *bes1-D*, *bzr1-D* and *bsu1-D*, also showed enhanced sensitivity to ABA-mediated inhibition of root growth. Triple loss-of-function mutants affected in PP2Cs that act as negative regulators of ABA signaling showed impaired root growth in the absence of exogenous ABA, indicating that disturbed regulation of ABA sensitivity impairs root growth. In agreement with this result, diminishing ABA-sensitivity of *brx-2* by crossing it with a *35S:HAB1* ABA-insensitive line allowed significantly higher recovery of root growth after BL treatment. Finally, transcriptomic analysis revealed that ABA treatment negatively affects auxin signaling in wt and *brx-2* roots and that ABA response is globally altered in *brx*-2. Taken together, our results reveal an interaction between BRs, auxin and ABA in the control of root growth and indicate that altered sensitivity to ABA is partly responsible for the *brx* short root phenotype.

Introduction

Abscisic acid (ABA) is a universal stress hormone of higher plants that also plays a key role as a regulator of growth and meristem function and in different plant developmental processes, such as embryo development, germination, vegetative development, flowering and organogenesis (Xu et al., 1998; Finkelstein et al., 2002; Barrero et al., 2005; Razem et al., 2006; de Smet et al., 2006; Liang et al., 2007). Both positive and negative effects of ABA on growth and development have been reported depending on tissue, concentration and interaction with the environment (Zeevaart and Creelman, 1988; Thompson et al., 2007). For instance, in tomato and *Arabidopsis*, normal levels of ABA are required to maintain shoot-growth independently of effects of

hormone status on plant water balance (Sharp et al., 2000; LeNoble et al., 2004). ABA inhibits germination and root growth at micromolar concentration, whereas low concentrations of ABA (<1 μM) stimulate root growth (Zeevaart and Creelman, 1998; Ephritikhine et al., 1999). This fact likely explains the variable effects on root growth obtained after exogenous application of ABA in well-watered plants, ranging from growth inhibition or little effect to growth promotion. Finally, a crucial ABA-dependent adaptive feature that promotes survival of plants under water stress is the maintenance of root elongation (Sharp et al., 2004). The manipulation of endogenous ABA levels by either chemical or genetic means has shown that ABA is crucial to maintain primary root growth at low water potentials (Saab et al., 1990). In contrast, the formation of a lateral root from a lateral root primordium is repressed as water availability is reduced, and ABA is a critical component of this repression mechanism (Deak and Malamy, 2005; De Smet et al., 2006). Finally, the importance of ABA on root growth control and root system architecture is reflected by the recent identification of a major QTL in maize that simultaneously affects ABA biosynthesis and root agronomical traits both under well-watered and water-stress conditions (Landi et al., 2007).

Plant growth and development are controlled by the concerted action of many signaling pathways, which integrate information from the environment with that from developmental and metabolic cues. In the particular case of root development, genetic analysis indicates that hormone signaling pathways functionally intersect with each other for the control of root growth. For instance, auxin controls the growth of roots by modulating cellular responses to the phytohormone gibberellin (Fu and Harberd, 2003). In this case, shoot-apex derived auxin controls root growth through modulation of GA-mediated DELLA protein destabilization (Fu and Harberd, 2003). Auxin signaling itself shows interdependency with BR signaling (Nemhauser et al., 2004; Nakamura et al., 2006, Hardtke et al., 2007). Other phyothormones (ethylene, cytokinin, ABA) interact in the regulation of plant growth and development, however the molecular mechanisms of these interactions remain poorly understood. Hormone response mutants have been of crucial importance to dissect the signal transduction pathways that control diverse physiological processes as well as genetic interactions among different signaling pathways (Gazzarrini and McCourt, 2001). Thus, interactions of ABA with signaling pathways of drought, salinity, cold, sugars, gibberellins, jasmonic acid, pathogenic elicitors, auxins, ethylene and brassinosteroids (BRs) have been described (Thomashow, 1999; Ghassemian et al., 2000; Beaudoin et al., 2000; Steber and McCourt, 2001; Cheng et al., 2002; Zhu, 2002; Finkelstein et al., 2002; Rock and Sun,

2005; Torres-Zabala et al., 2007; Adie et al., 2007). In particular, ABA and BRs have been reported to act antagonistically in some plant responses. For instance, BRs promote whereas ABA inhibits germination, and both the BR biosynthetic mutant *det2-1* and the BR-insensitive mutant *bri1-1* are more sensitive than wild type to ABA-mediated inhibition of germination (Steber and McCourt, 2001). Additionally, both BR biosynthetic and perception mutants are hypersensitive to ABA-mediated inhibition of root growth (Clouse et al., 1996; Ephritikhine et al., 1999). Finally, the expression of the BR ENHANCED EXPRESSION BEE1, BEE2 and BEE3 transcription factors was repressed by ABA, and *BEE1* over-expressing roots were hypersensitive to BRs and partially insensitive to ABA (Friedrichsen et al., 2002). However, good candidates that could explain the ABA-BR crosstalk at the molecular level have not been identified.

To further extend our knowledge on the ABA signaling pathway and its effect on growth regulation, we have performed a screen for mutants hypersensitive to ABA in growth assays. As a result, we have identified a mutant in Columbia background, named *sha1*, which showed enhanced sensitivity to ABA-mediated inhibition of root growth and was found to be allelic to the previously identified *brevis radix* (*brx*) mutant. *BRX* is a key regulator of cell proliferation and elongation in the root, which is expressed in the phloem vasculature throughout the plant (Mouchel et al., 2006). BRX has been implicated in the interaction between the auxin and BR pathways based on the observation that in *brx* mutant globally impaired auxin-responsive gene expression can be rescued by BR application (Mouchel et al., 2006). Exogenous application of brassinolide (BL) also partially rescued the root growth defect of *brx-1* (from ~30% root length of wt control to >50%) (Mouchel et al., 2006). Constitutive expression of a rate limiting enzyme in BR biosynthesis, CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARF (CPD), in *brx-1* driven by the CaMV *35S* promoter was slightly more efficient (~60% of wt control) to rescue this phenotype (Mouchel et al., 2006). Our finding of *brx-2* as hypersensitive to ABA-mediated inhibition of root growth reveals a novel phenotype for this mutant and suggests that *BRX* and BRs play important roles in modulating root response to ABA. This is corroborated by analysis of other mutants in the BR signaling pathway. Taken together, our results reveal a cross-talk between ABA and BRs for root growth control and suggest that a normal response to BRs is required to prevent enhanced sensitivity to ABA-mediated inhibition of root growth.

Results

brx-2 loss-of-function mutant shows enhanced sensitivity to ABA-mediated inhibition of root growth

A screen for mutants hypersensitive to ABA-mediated growth inhibition was performed using T-DNA lines generated with the activation-tagging vector pSKI15 in a Columbia background. Seeds were germinated vertically in the absence of ABA and then 5-day-old seedlings were transferred to plates supplemented with 30 μM ABA. Potential ABA-hypersensitive mutants were initially identified on the basis of impaired growth compared to wild type. After screening of approximately 20000 lines, several candidates were selected and initially named *seedling hypersensitive to ABA* (*sha*) mutants. In the absence of ABA, the *sha1* mutant showed a slight decrease in root growth at 5 days compared to wt, which was notably increased at further stages of development (Fig. 1A). As it is discussed below, *sha1* was found to be allelic to *brx* and accordingly, we renamed it as *brx-2*. The presence of 10 μM ABA in the medium exacerbated the root growth defect in *brx-2* compared to wt (73% and 45% inhibition, respectively) (Figure 1A and 1B). Interestingly, the double *hy5 hyh* mutant, which displays reduced root growth because of reduced cell proliferation in the meristem (Sibout et al., 2006), did not show enhanced ABA-mediated inhibition of root growth (Figure 1B), suggesting that the observed ABA hypersensitivity of *brx-2* does not simply reflect disproportional growth reduction due to already initially impaired root growth. Both ABA-mediated inhibition of germination and water loss kinetics were similar in *brx-2* and wild type, in contrast to the global ABA-hypersensitive phenotype of the double *hab1-1 abi1-2* mutant (Saez et al., 2006) (Fig 1C; Supplemental Fig. S1).

The mutation was recessive because F1 seedlings showed similar root growth than wild type both in the absence and presence of ABA (data not shown). The segregation ratio in the F2 progeny was consistent with a single, recessive mutation (132 wild type: 46 short root phenotype; $\chi 2=0.067$, P=0.79). Homozygous *sha1* individuals were selected from the F2 generation and scored for phosphinothricin resistance. All F2 *sha1* seedlings showed co-segregation of the *sha1* phenotype and phosphinothricin resistance. Plant T-DNA flanking sequences were isolated from the mutant by TAIL-PCR and sequence analysis revealed that the pSKI15 T-DNA was inserted at nucleotide 553 of the *BREVIS RADIX* (*BRX*, *At1g31880*) gene (Fig. 1D).

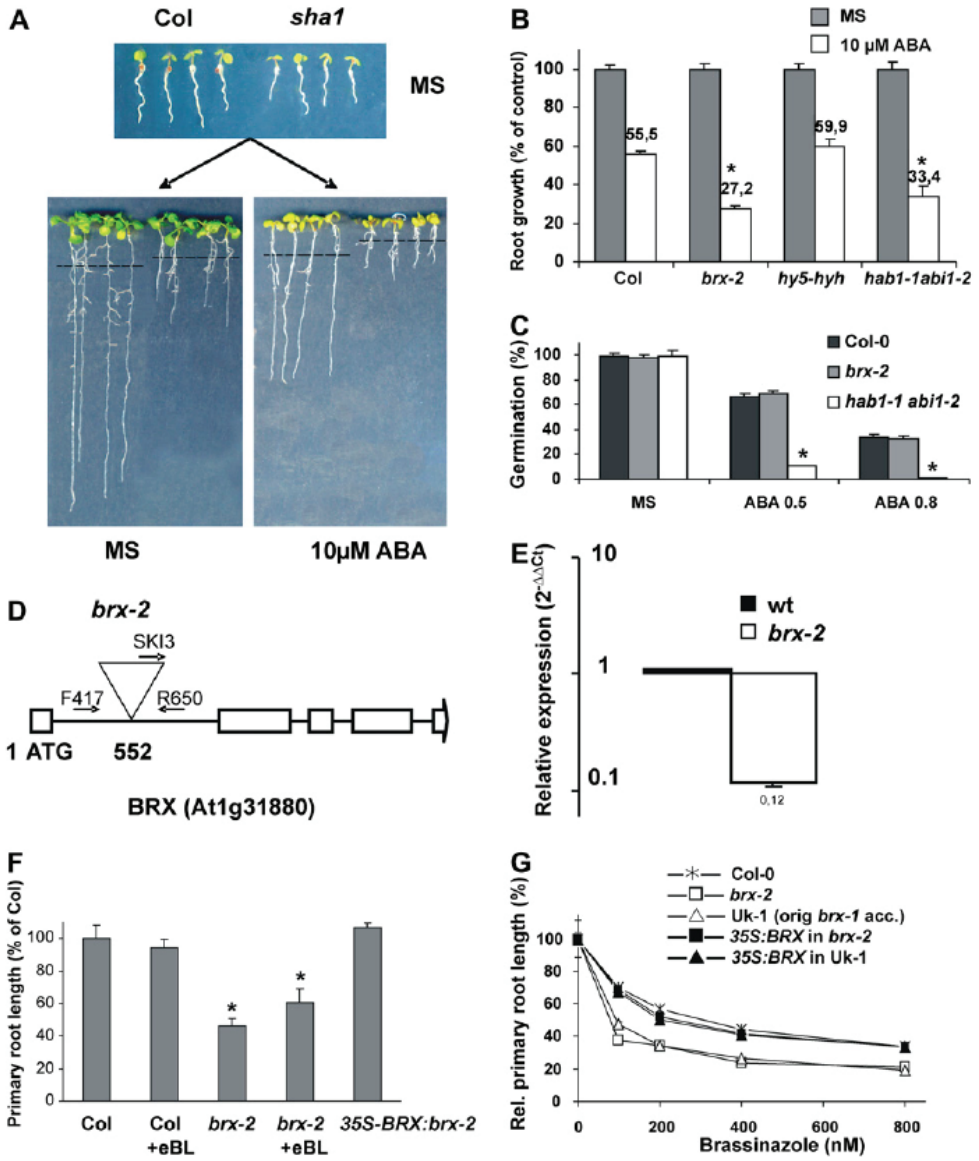

Figure 1. *brx-2* shows higher sensitivity than wild type to ABA-mediated root growth inhibition. A, Five-day-old seedlings (top panel) were transferred to medium lacking or supplemented with 10 µM ABA (lower panels) and grown vertically for 6 days. The dotted line indicates the position of root tip just after transferring to new medium. B, Quantification of root growth inhibition by ABA in wild type, *brx-2*, double *hy5hyh* and *hab1-1abi1-2* mutants. Data are averages ±SE from three independent experiments (n=20). C, *brx-2* shows similar sensitivity than wild type to ABA-mediated inhibition of germination. Data are averages ±SE from three independent experiments (n=200). D, Scheme of *BRX* gene and localization of T-DNA insertion in *brx-2*. The numbering begins at the ATG translation start codon. Primers used for co-segregation analysis are indicated. E, RT-qPCR analysis of *BRX* expression in wild type and *brx-2* mRNAs prepared from 2-week-old roots. Values are relative expression levels with respect to Col (value 1). Data are averages ±SE from three independent experiments. F, Relative root length of seedlings from wt (Col ecotype), *brx-2* and complemented line (*35S-BRX*::*brx-2*) grown vertically in medium lacking or supplemented with BL. Five-day-old seedlings were transferred to medium lacking or supplemented with 2 nM brassinolide (BL) and grown vertically for 5 days. Root length of wild type in the absence of exogenous BL was taken as 100%. Data are averages ±SE from three independent experiments (n=20). G, Brassinazole-hypersensitivity of *brx-1* (natural allele in Uk-1 accession), *brx-2* and complemented lines (*35S-BRX*::*brx-2; 35S-BRX* in Uk-1) in root growth assays. Data are averages from three independent experiments (n=20) (SE <3%, SE bars are not visible due to overlapping with legend marks). *, $P<0.05$ (Student's *t* test) when comparing data from the indicated genotype and WT in the same growth conditions.

A natural loss-of-function allele of *BRX* in the *Arabidopsis* accession Umkirch-1 (Uk-1) had been previously reported (Mouchel et al., 2004), which we name as *brx-1*, and therefore we have renamed *sha1* as *brx-2*. Finally, the analysis of 52 F2 *brx-2* chromosomes showed co-segregation of the short root phenotype and the presence of the T-DNA (data not shown). Real-time quantitative PCR (RT-qPCR) analysis using primers that amplify the 3´-end of *BRX* cDNA showed that the T-DNA insertion found in *brx-2* strongly impaired the expression of *BRX* (Fig. 1E).

In *brx-1* mutant, exogenous application of brassinolide (BL) partially rescued the root growth defect, whereas introduction of a 35S:*BRX* transgene fully restored root growth (Mouchel et al., 2006). Similar chemical and genetic complementation assays were performed with *brx-2*, and analogous results were obtained (Fig. 1F). Finally, *brx-2* also resembles the original allele in its hypersensitivity to root growth inhibition by brassinazole, a BL biosynthesis inhibitor (Fig. 1G).

Roots of *bes1-D*, *bzr1-D* and *bsu1-D* mutants are ABA-hypersensitive

Although the biochemical function of BRX has not been elucidated yet, the fact that it can localize to the nucleus and its ability to activate transcription in a heterologous yeast system, have led to the suggestion that BRX might represent a novel class of transcriptional regulator (Mouchel et al., 2004). We wondered whether other transcription factors that directly affect BR action might show an ABA-hypersensitive root phenotype as well. BES1 and BZR1 are a novel class of plant-specific transcription factors that play a key role in BR signaling (Yin et al., 2002 and 2005; Wang et al., 2002; He et al., 2005). Therefore, we examined ABA sensitivity of *bes1-D* and *bzr1-D* mutants in root growth assays and we compared these results with *brx-2* as well as the ABA-hypersensitive double *hab1-1 abi1-2* mutant and ABA-insensitive *35S:HAB1* plants (Saez et al., 2004 and 2006) (Fig. 2). Interestingly, the *bes1-D* mutant showed a strong ABA-hypersensitive phenotype and, in contrast to *brx*, was not impaired in root growth in the absence of ABA. The *bzr1-D* mutant showed a weak ABA-hypersensitive phenotype at 1 and 3 μM ABA, and a stronger phenotype at 10 μM ABA (Fig. 2B). The nuclear protein phosphatase BSU1 is able to modulate the phosphorylation state of BES1 (Mora-García et al., 2004). The gain-of-function *bsu1-D* mutation leads to increased steady-state levels of dephosphorylated BES1 and thereby, modulates the magnitude of the response to BR (Mora-García et al., 2004). Interestingly, the *bsu1-D* mutant was as hypersensitive to ABA-mediated root growth inhibition as *bes1-D* (Fig.

2). It is noteworthy that all *bes1-D*, *bzr1-D* and *bsu1-D* mutants show enhanced ABA-mediated inhibition of root growth even though they are not short-rooted mutants. Taken together, these results show that modulation of plant response to BR strongly affects ABA sensitivity of roots.

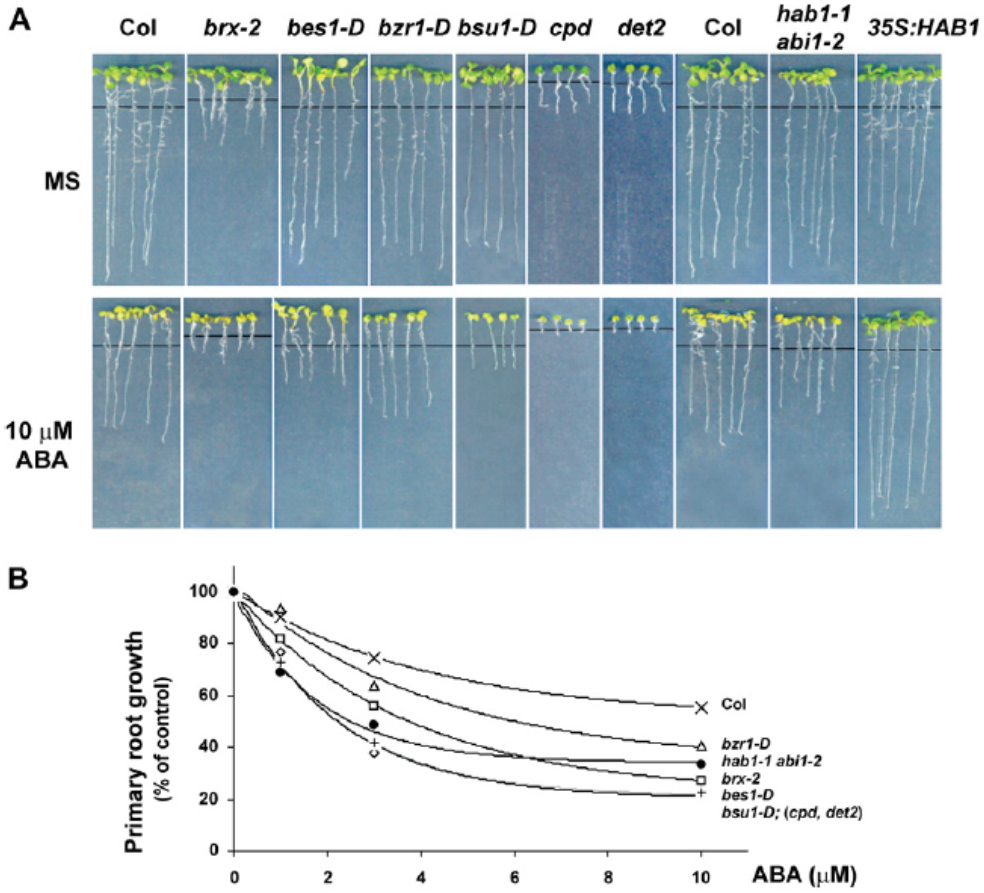

Figure 2. *bes1-D*, *bzr1-D*, *bsu1-D*, *cpd* and *det2* mutants show higher sensitivity than wild type to ABA-mediated root growth inhibition. A, Four-day-old seedlings were transferred to medium lacking or supplemented with 10 μM ABA and grown vertically for 6 days. B, Quantification of root growth inhibition by 1, 3 and 10 μM ABA in the indicated genotype. Data are averages from three independent experiments (n=20) and are expressed as percentage of root growth with respect to each genotype in the absence of ABA (SE <4%, bars are not visible due to overlapping with legend marks). *cpd* and *det2* graphics (not shown) overlap to those of *bes1-D* and *bsu1-D*.

Triple *pp2c* loss-of-function mutants show extreme ABA-hypersensitivity and impaired root growth

Root sensitivity to ABA must be finely tuned to properly respond to changing environmental conditions and to prevent negative effects of ABA on root growth under well-watered conditions. As *brx-2* shows both a defect in root growth and enhanced

sensitivity to ABA, we wondered whether mutants showing a hypersensitive response to ABA might be impaired in root growth. To answer this question, we have generated mutants that present different degrees of ABA-hypersensitivity through combination (single, double and triple) of loss-of-function mutations in the PP2Cs that act as negative regulators of ABA signaling (Saez et al., 2004; Saez et al., 2006) (Figure 3A).

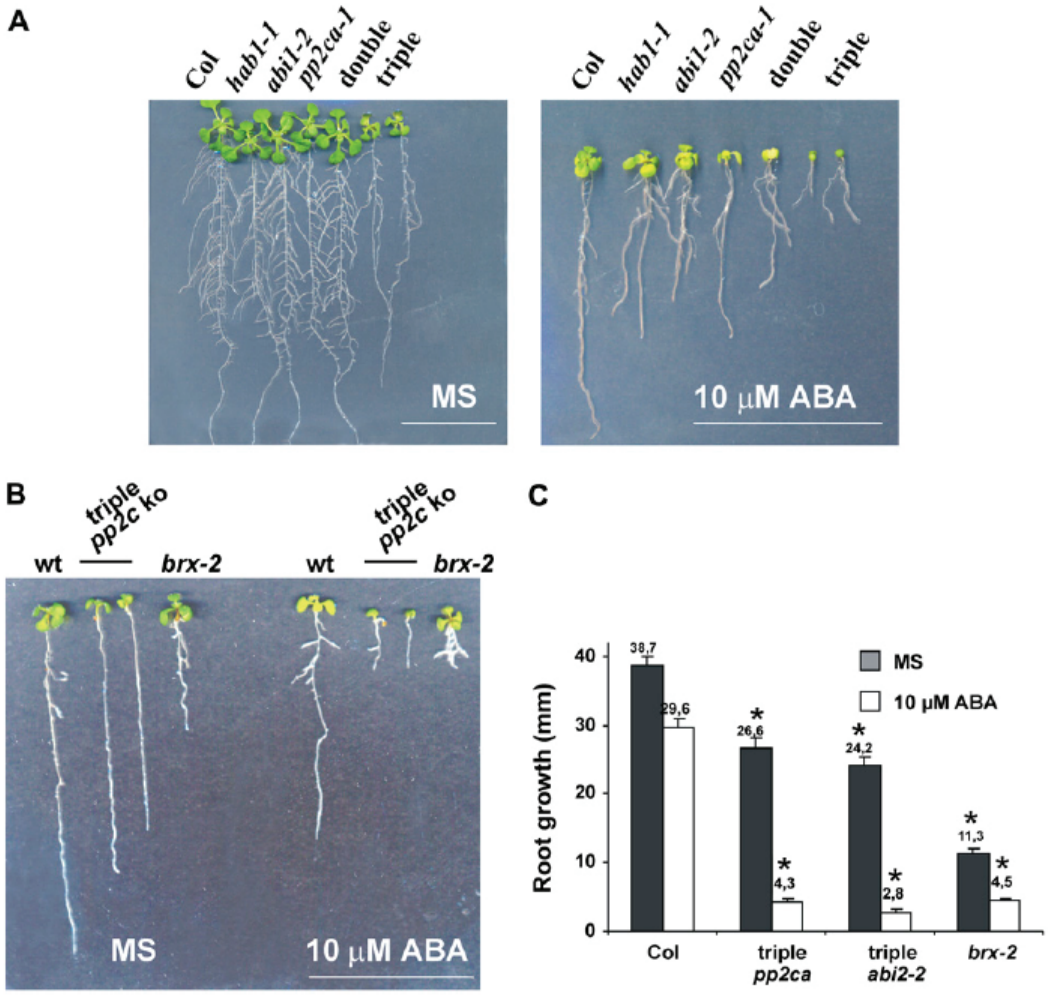

Figure 3. Impaired root growth of triple *pp2c* knockout (ko) mutants. A and B, Growth of wild type and ABA-hypersensitive mutants *hab1-1*, *abi1-2*, double *hab1-1abi1-2* and triple *hab1-1abi1-2abi2-2* and *hab1-1 abi1-2 pp2ca-1* mutants (triple *pp2c* ko) in medium lacking or supplemented with 10 μM ABA. Scale bars correspond to 3 cm. The photographs were taken after 12 (A) or 6 (B) days of the transfer of 5-d-old seedlings from MS medium to plates containing 10 μM ABA. C, Quantification of root growth for Col wild type (wt), triple *pp2c* ko and *brx-2* after 6 d in MS medium lacking or supplemented with 10 μM ABA Data are averages ±SE from three independent experiments (n=20); *, *P*<0.05 (Student's *t* test) when comparing data from each genotype to wt in the same growth conditions.

Thus, *hab1-1 abi1-2 pp2ca-1* and *hab1-1 abi1-2 abi2-2* triple mutants were generated and real-time quantitative (RT-qPCR) analyses confirmed that expression of *HAB1*, *ABI1* and either *PP2CA* or *ABI2*, respectively, was severely impaired (Supplemental Fig. S2). Whereas single and double *pp2c* mutants did not show a defect in root growth in the absence of exogenous ABA (Saez et al., 2004; Saez et al., 2006), both triple *hab1-1 abi1-2 abi2-2* and *hab1-1 abi1-2 pp2ca-1* mutants were impaired in root growth, although less severely than *brx-2* (Fig. 3). Both triple mutants showed a constitutive transcriptional response to endogenous ABA levels (data not shown) and were extremely hypersensitive to exogenous addition of ABA in assays of root growth inhibition (Fig. 3). These results suggest that negative regulation of ABA signaling by PP2Cs is required to prevent inhibition of root growth by endogenous ABA levels.

Introduction of ABA insensitivity into *brx-2* improves root growth rescue by BL treatment

Exogenous application of BL partially rescued the root growth defect of both *brx-1* and *brx-2* (Mouchel et al., 2006) (Figure 1F). Taking into account the root phenotype found in triple *pp2c* mutants, we reasoned that the enhanced ABA sensitivity of *brx-2* might prevent a better rescue of its root growth defect by BL. To challenge this hypothesis, we conferred ABA insensitivity to *brx-2* by crossing it with a *35S-HAB1* transgenic line (Saez et al., 2004). Over-expression of the PP2C HAB1, which is a negative regulator of ABA signaling, leads to reduced sensitivity to ABA as compared to wild type (Saez et al., 2004). F2 *brx-2* individuals that showed reduced sensitivity to ABA because of the presence of the *35S-HAB1* construct, and consequently enhanced expression levels of HAB1 (Figure 4C), were selected and their root growth in response to BL was analysed. Figure 4 shows that 5 days after transfer onto BL media, root growth in *brx-2* was enhanced to >120% of control length. By contrast, recovery of root growth to >150% of control length was achieved in *35S-HAB*1::*brx-2* lines. Importantly, BL treatment did not enhance root growth of *35S-HAB1* lines. These results suggest that disturbed regulation of ABA sensitivity in *brx-2* prevents full complementation of the root growth defect by exogenous addition of BL.

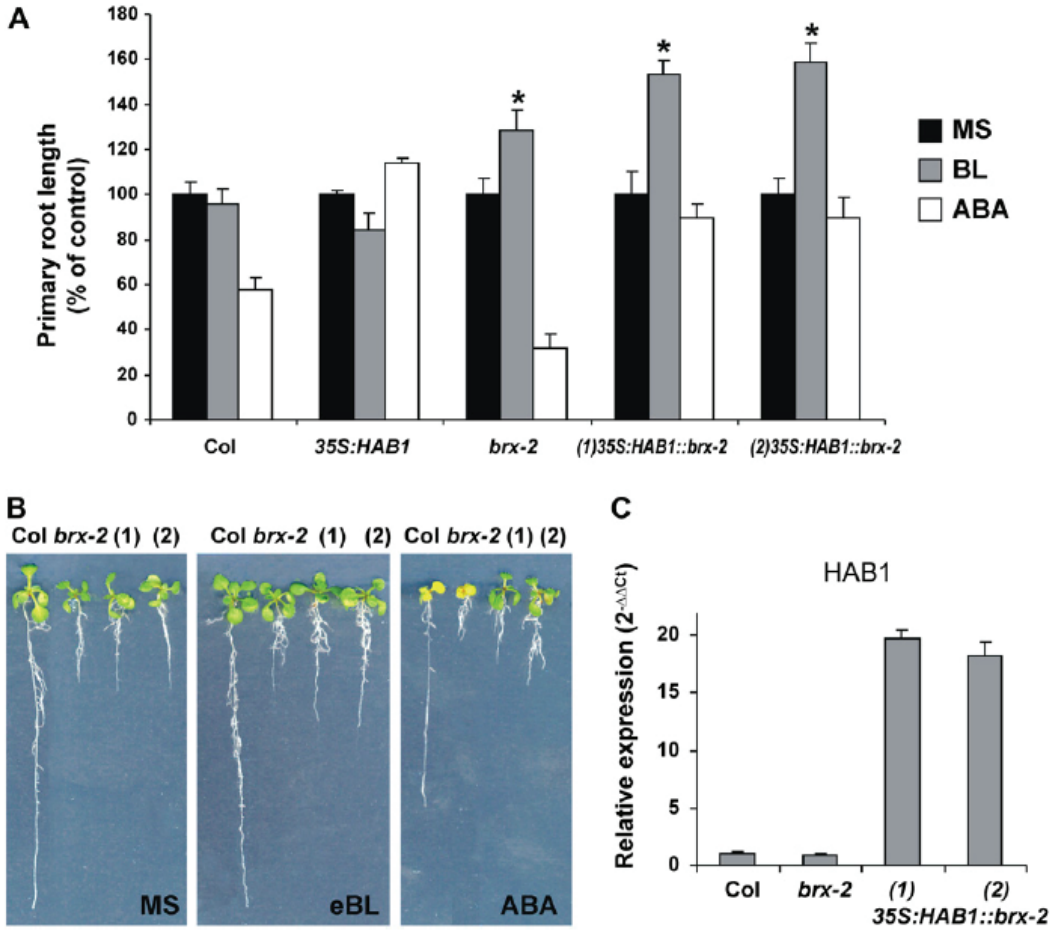

Figure 4. Improved root growth rescue by brassinolide (BL) in *35S-HAB1*::*brx*-2. A, Relative root length of seedlings from wt, *35S-HAB1* line, *brx-2* and two representative transgenic lines *35S-HAB1*::*brx-2*. Seeds were germinated on MS medium and four-day-old seedlings were transferred to new MS plates lacking or containing either 2 nM BL or 10 μM ABA and grown in vertical position for 6 days. Data are averages ±SE from three independent experiments (n=20). * indicates P<0.05 (Student´s *t* test) with respect to the same genotype in MS medium. B, Photographs of representative seedlings after five days of vertical growth in MS medium lacking or supplemented with either 2 nM brassinolide (BL) or 10 μM ABA. C, RT-qPCR analysis of *HAB1* expression in wt, *brx-2* and two transgenic lines *35S-HAB1::brx-2*. Values are relative expression levels with respect to Col (value 1). Data are averages ±SE from three independent experiments.

Transcriptomic analysis of ABA-response in roots of *brx-2* compared to wild type and *cpd* mutant

To further investigate the role of *BRX* in the modulation of root sensitivity to ABA, transcriptomic profiles of wild type and *brx-2* were obtained from mock- or ABA-treated roots. Whole-genome long-oligonucleotide microarrays were used to compare ABA-mediated upregulation/downregulation of gene expression in wild type and *brx-2* (Figure 5A). The overlap of ABA-upregulated genes (ratio of expression >2-fold, false

discovery rate p<0.05) in wild type and *brx-2* was approximately 75%. Among these genes, 507 out of 1727 were identified as differentially expressed in wild type and *brx-2* (Figure 5B): 250 genes were upregulated by ABA to a greater extent in the *brx-2* mutant than in wild type, whereas 257 genes showed higher induction by ABA in wild type than *brx-2* (Figure 5B). With respect to ABA-downregulated genes (ratio of expression <0.5-fold, false discovery rate p<0.05), *brx-2* showed a higher number of affected genes, 1836, than wild type, 1396, and the overlap between those gene sets was 996 (Figure 5A). Among them, 309 out of 996 showed differential expression between wild type and *brx-2*: 165 genes were downregulated by ABA to a greater extent in *brx-2* than in wild type, whereas 144 genes were more downregulated in wild type than in *brx-2* (Figure 5B). Therefore, approximately 30% of genes that were ABA-responsive (upregulated or downregulated) in both wild type and *brx-2* showed differential expression between the two genotypes. Taken together, these data reveal a globally altered transcriptional response to ABA in *brx-2* compared to wild type. Specifically, 250 and 166 genes showed enhanced ABA-mediated upregulation or downregulation, respectively, in *brx-2* compared to wild type. However, some of these changes might be due to impaired expression of *CPD* in *brx* background, and hence reduced BR biosynthesis (Mouchel et al., 2006). Indeed, *cpd* mutant is very hypersensitive to ABA-mediated inhibition of root growth (Figure 2A). To further explore this possibility we have obtained the root transcriptomic profile of *brx*+ABA compared to *cpd*+ABA. Genes that showed enhanced response to ABA in *brx-2* compared to wt were divided in two groups according to the ratio of expression *brx-2* + ABA/ cpd + ABA (Figure 5B). Thus, when this ratio was comprised between 0.5 and 2, we considered that the observed changes in ABA response represented an indirect effect of impaired *CPD* expression in *brx* background. This first group comprised ~60-80% of the selected genes. However, a significant proportion of genes that showed enhanced response to ABA in *brx-2* compared to wt represented a direct consequence of the *brx-2* mutation because the ratio of expression *brx-2* + ABA/ cpd + ABA was either >2 or, to lesser extent, <0.5. Using this criterion, we found that 20% and 38% of genes showing enhanced ABA-mediated upregulation or downregulation (in *brx-2* compared to wt), respectively, are a direct consequence of the *brx-2* mutation. Interestingly, among the former genes we identified several one encoding RING finger E3 ligases related to the SDIR1 protein, which is a positive regulator of ABA signaling since SDIR1 overexpression leads to ABA hypersensitivity (Zhang et al., 2007).

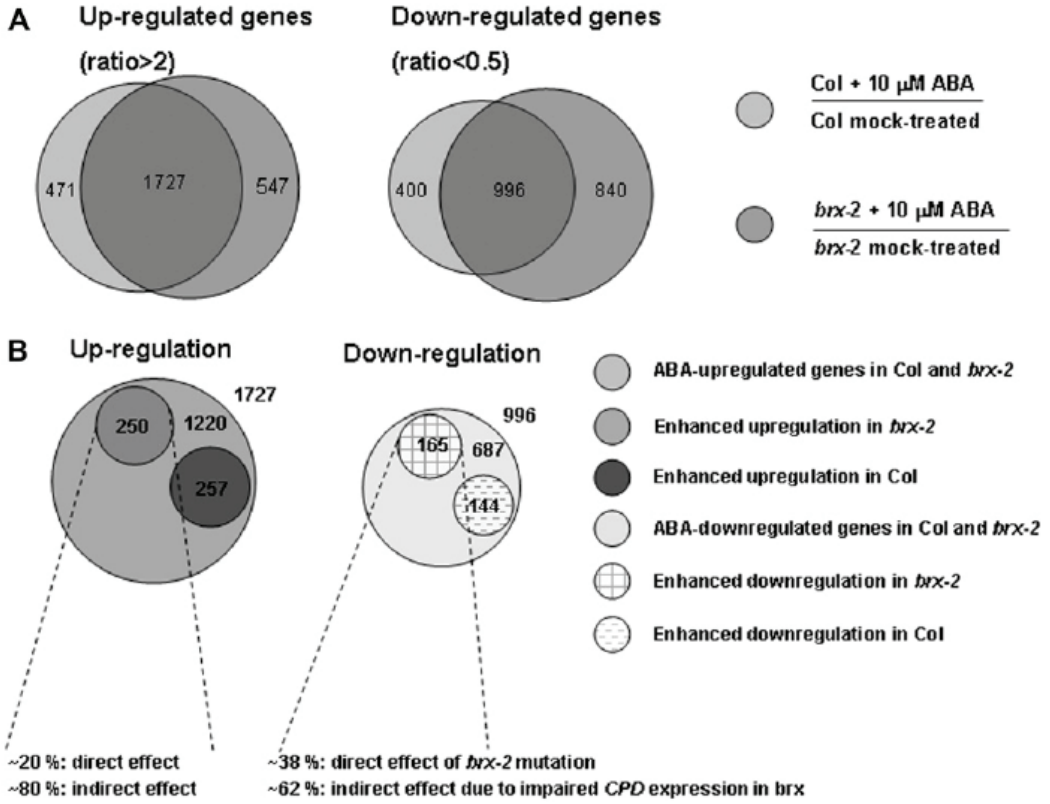

Figure 5. Transcriptomic analysis of ABA-response in roots of *brx-2* compared to wild type (Col) and *cpd* mutant. A, Total number of ABA-responsive genes in wt and *brx-2*. Threshold of 2-fold, ratio>2 or <0.5 for up- and down-regulated genes, respectively (false discovery rate p<0.05). B, Number of genes up- or down-regulated by ABA in both wt (Col) and *brx-2* that show a differential expression in *brx-2* compared to wild type (threshold of 1.4-fold according to SAM; Tusher et al., 2001). Genes that showed enhanced response to ABA in *brx-2* were divided in two groups (direct effect of *brx-2* mutation or indirect effect due to impaired *CPD* expression in *brx*) according to the ratio of expression *brx-2* + ABA/ *cpd* + ABA (see text for a detailed explanation).

The ABA-treatment of roots led to upregulation of genes involved in stress response: oxidative stress, osmotic, salt, heat shock and cold as well as LEA proteins. ABA-mediated upregulation of genes involved in hyperosmotic stress response might be beneficial under low water potential conditions, as ABA promotes growth under those conditions (Sharp et al., 2004). On the other hand, the antagonism suggested for ABA and auxin in development of root system architecture (Deak and Malamy, 2005; De Smet et al., 2006) was reflected in our transcriptomic analysis, as ABA-treatment induced the expression of several Aux/IAA repressor proteins (IAA3, IAA6, IAA11, IAA18, IAA28, IAA30), whereas it repressed several genes involved in auxin biosynthesis (Table I). This effect was observed in both wt and *brx-2*. However, taking

into account that auxin-responsive gene expression is globally impaired in *brx* (Mouchel et al., 2006), it is reasonable to postulate that further impairment of auxin response by ABA will affect *brx* more negatively than wt.

Category	AGI Code	Gene Nomenclature	Col + ABA / Col mock	*brx-2* + ABA / *brx-2* mock
Aux/IAA genes	AT4G28640	IAA11	3.8	3.1
	AT5G25890	IAA28	2.1	3.1
	AT1G51950	IAA18	2.8	2.6
	AT1G52830	IAA6	4.8	2.8
	AT1G04240	IAA3	3.5	4.1
	AT3G62100	IAA30	6.1	7.2
GH3 family				
	AT5G13360		3.0	3.5
	AT5G13370		15.6	16.1
Auxin transport				
	AT5G57090	PIN2	0.6	0.4
	AT2G47000	PGP4	1.4	0.4
	AT3G28860	PGP19	0.7	0.4
F-box protein TIR1 family				
	AT4G03190	AFB1	0.6	0.4
Auxin biosynthesis				
	AT5G05730	ASA1,AMT1	0.2	0.4
	AT1G25220	TRP4	0.4	0.4
	AT3G54640	TRP3	0.2	0.4
	AT2G20610	ALF, HLS3, RTY, SUR1	0.2	0.3

Table I. List of genes involved in auxin action that are up- or down-regulated in *brx-2* (threshold >2, ratio>2 or <0.5 for up- and down-regulated genes, respectively, false discovery rate p<0.05).

Discussion

In this work, starting from the isolation of *brx-2* as an ABA-hypersensitive mutant in root growth assays, we provide evidence for a role of BRX as a modulator of ABA sensitivity in roots. Importantly, the enhanced ABA response of *brx-2* was specific for the root, as ABA-mediated inhibition of seed germination and water loss kinetics were similar to wild type. Thus, it appears likely that *BRX* mediates a root-specific branch of the ABA signaling pathway. Alternatively, it is conceivable that other *BRX-like* genes (*BRXLs*) might have a role in ABA response that is masked by functional redundancy in the *brx-2* mutant. *BRX* is expressed in the columella and the phloem vasculature throughout root and shoot (Mouchel et al., 2006). Vascular expression of *BRX* was also detected in a torpedo stage embryo (Mouchel et al., 2006). However, global expression of *BRX* as well as *BRX-like* genes (*BRXL1*, *BRXL2*, *BRXL3*) is low, as demonstrated by their

relative expression compared to a housekeeping gene, *eIF4* (Mouchel et al., 2006), or embryo expression compared to *HAB1,* which plays an important role to control ABA-sensitivity in seed (Saez et al., 2004; Supplemental Fig. S3). Likely, *BRX* expression at stomata is low and expression of other *BRXLs* might lead to genetic redundancy and therefore lack of phenotype in transpiration assays (Supplemental Fig. S3).

As BRX has been previously implicated in connecting the auxin and brassinosteroid pathways, our results suggest an interaction between the auxin and/or BR and ABA pathways in root development. For the BR pathway, we tested this notion directly by investigating whether other mutants affected in BR action show altered sensitivity to ABA in root growth assays. Indeed, *sax1* (Ephritikhine et al., 1999) and *det2* (Figure 2), which are defective in BR biosynthesis, and *bri1-1* (Clouse et al., 1996), defective in BR signaling, are impaired in root growth and extremely hypersensitive to ABA-mediated inhibition. Paradoxically, an enhanced response to BR also increased the inhibitory effect of ABA on root growth, as *bzr1-D*, *bes1-D* or *bsu1-D* show constitutive BR response and were hypersensitive to ABA-mediated root growth inhibition. Therefore, a similar morphological response to ABA is generated when BR action is disturbed because of a BR biosynthetic/signaling defect or through mutations that lead to constitutive BR response phenotypes. These results suggest that homeostatic control of BR signaling is required for a normal response to ABA. Alternatively, it has been demonstrated that BZR1, in addition of being a positive regulator of the BR signaling pathway, also mediates negative feedback regulation of BR biosynthesis (Wang et al., 2002). Likewise, in-depth analysis of *bes1-D* microarray data reveals that several biosynthetic genes are down-regulated in this mutant (Vert et al., 2005). Therefore, it is possible that the lower BR levels found in *bzr1-D* and, presumably, *bes1-D* might be responsible of their ABA-hypersensitive phenotype. Nevertheless, the fact that *bes1-D*, *bzr1-D* and *bsu1-D* are all ABA-hypersensitive but, unlike BR biosynthesis mutants or *bri1*, do not display a defect in root growth supports the idea that a branch of the BR signaling pathway directly impinges on ABA response. Our striking observation that introduction of ABA-insensitivity into the *brx-2* background significantly enhances the rescue of the short root phenotype by exogenous BL treatment supports this idea. It will be interesting to see whether the phenotype of other genuine BR signaling mutants like *bri1* can be partially healed in a similar fashion.

Conversely, one might expect that impairment of the ABA signaling pathway would yield a root growth phenotype. Indeed, the fact that enhanced sensitivity to ABA impairs root growth appeared to be masked by genetic redundancy so far, as

demonstrated by the phenotype of triple knockouts impaired in some of the PP2Cs that act as negative regulators of ABA signaling (Saez et al., 2006). Both triple *hab1-1 abi1-2 abi2-2* and *hab1-1 abi1-2 pp2ca-1* mutants were extremely sensitive to the inhibitory effect of ABA on root growth and, interestingly, root growth was notably reduced in the triple mutants in control conditions as compared to wild type. Taken together, both our physiological and phenotypic analyses reveal that disturbed regulation of root ABA sensitivity leads to inhibition of root growth.

With respect to regulation of root growth, it has been suggested that BR biosynthesis and auxin signaling are connected through a feedback mechanism that involves BRX. Transcriptomic analysis showed that impaired auxin-responsive gene expression in the *brx-1* mutant could be restored by BL application, indicating feedback between BR levels and auxin signaling in root growth (Mouchel et al., 2006). ABA treatment in roots of wild type and *brx-2* led to upregulation of Aux/IAA repressor genes as well as downregulation of genes involved in auxin biosynthesis (Table I). Aux/IAA repressor proteins are known to be negative regulators of auxin signaling through dimerization with auxin response factors (ARFs), as Aux/IAA proteins prevent ARFs from promoting transcription of auxin-responsive genes (Tiwari et al., 2001 and 2003). Therefore, ABA-treatment had a negative effect on auxin signaling in roots. This effect was observed in both wt and *brx-2*. However, taking into account that auxin-responsive gene expression is globally impaired in *brx* (Mouchel et al., 2006), it is reasonable to suggest that further impairment of auxin response by ABA will have a stronger effect on root growth in *brx* than in wt. This effect might partially explain why *brx-2* is more sensitive to the inhibitory effect of ABA on root growth. Additionally, the differential ABA-mediated up-regulation of RING finger E3 ligases related to the SDIR1 protein in roots of *brx-2*, might also contribute to the enhancement of ABA response. Finally, a direct comparison of transcriptomic response to ABA in wild type and *brx-2*, revealed that approximately 30% of ABA-upregulated and ABA-downregulated genes were differentially expressed in wild type and *brx-2*. These results suggest that BRX has an important role in regulating transcription in response to ABA. Thus, it appears likely that enhanced stress perception due to impaired ABA response is not only partly responsible for the short root phenotype, but also for the pronounced perturbation of the transcriptome in *brx* mutants.

Finally, our analyses of BR biosynthesis and signaling mutants suggest that enhanced ABA and thus stress perception might significantly contribute to the root phenotypes of various hormone pathway mutants. Such perturbation of ABA sensitivity

might be variable depending on the context and the level at which a given pathway is interrupted, but could explain the seemingly disparate root growth phenotypes of signaling mutants that have been shown to reside in the same pathway. Future analyses using the transgenic approach described here might help to clarify whether this is indeed the case.

Materials and methods

Plant Material and Growth Conditions

Arabidopsis plants were routinely grown under greenhouse conditions in pots containing a 1:3 vermiculite-soil mixture. For in vitro culture, seeds were surface sterilized by treatment with 70% ethanol containing 0.1% Triton X-100 for 20 min, followed by four washes with sterile distilled water. After stratification in the dark at 4ºC for 2 d, seeds were sowed on plates containing Murashige-Skoog (MS) medium with 0.1% 2-[N-morpholino]ethanesulfonic acid, 1% agar, and 1% sucrose. The pH was adjusted to 5.7 with potassium hydroxide before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 22ºC under a 16-h light, 8-h dark photoperiod at 80 to 100 μE m^{-2} sec^{-1}.

Screening conditions

T-DNA lines were constructed in D. Weigel and C. Somerville laboratories using the pSKI15 vector, which encodes a phosphinothricin resistance gene (BAR driven by 5´pMAS). Approximately 86000 independent lines, stock numbers N21995, N21991, N23153 and N31100, were provided by the *Arabidopsis* Biological Resource Center (ABRC, Ohio, USA). After surface sterilization, seeds were sowed and grown on vertically oriented plates containing MS medium. After 5 days, seedlings were transferred to plates containing MS medium supplemented with 30 µM ABA. Potential ABA-hypersensitive mutants were selected after 5 days and left 2 days in MS medium for recovery, and finally they were transferred to soil.

Genetic analysis

The backcross of *sha1* mutant to Columbia wild type was performed by transferring pollen to the stigmas of emasculated flowers. F1 and F2 seedlings were scored for root growth in the absence or presence of exogenous ABA. From the segregating F2 generation, homozygous *brx-2* individuals were selected and DNA was individually

extracted to perform a co-segregation analysis between the *BRX* T-DNA insertion and the short root phenotype. To this end, the following primers were used: F417, 5´-GTCAGTGTTTGCTTCCTCTCTATG, R650, 5´- TATTTCCTTGTCTA GGTAAGAATCC and SKI3, 5´-TGATCCATGTAGATTTCCCGGACA TGAA. Additionally, the analysis of F2 *sha1* seedlings revealed co-segregation between the *sha1* phenotype and phosphinothricin resistance.

Generation of triple *pp2c* loss-of-function mutants

The double *hab1-1abi1-2* mutant has been previously described (Saez et al., 2006). Lines carrying T-DNA insertions either in ABI2 (SALK_015166, *abi2-2* allele) or PP2CA (SALK_028132, *pp2ca-1* allele) were identified in the SALK T-DNA collection (Alonso et al., 2003) and homozygous mutants were kindly provided by Dr Julian Schroeder. To generate the triple *hab1-1 abi1-2 abi2-2* and *hab1-1 abi1-2 pp2ca-1* mutants, we transferred pollen of either *abi2-2* or *pp2ca-1* to the stigmas of emasculated flowers of the double *hab1-1abi1-2* mutant. The resulting F2 individuals were genotyped by PCR in order to identify the triple mutants.

Thermal asymmetric interlaced PCR (TAIL-PCR)

DNA was obtained either through a CTAB-based isolation procedure or using the DNAeasy Plant Minikit (Qiagen). DNA samples were treated with RNase, extracted with phenol:chloroform:isoamylalcohol and ethanol-sodium acetate precipitated. Plant T-DNA flanking sequences were amplified by PCR according to the protocols of Liu et al., (2005). To this end the following primers were used: SKI1, 5´-AATTGGTAATTACT CTTTCTTTTCCTCCATATTGA; SKI2, 5´-ATATTGACCATCATACTCATTGCTGATC CAT; SKI3, 5´-TGATCCA TGTAGATTTCCCGGACATGAA; AD1, 5´-TG(AT)G(ACGT)AG(GC)A (ACGT)CA(GC)AGA; AD2, 5´-(ACGT)TCGA(GC)T(AT) T(GC)G(AT)G TT; AD3, 5´-(ACGT)GTCGA(GC)(AT)GA(ACGT)A(AT)GAA; AD4, 5´-AG(AT)G(ACGT)AG(AT)A(ACGT)CA(AT)AGG; AD5, 5´-(AT)GTG (ACGT)AG(AT)A (ACGT)CA(ACGT)AGA and AD6, 5´-(GC)TTG (ACGT)TA(GC)T(ACGT)CT(ACGT)TGC

Complementation of *brx-2*

BRX cDNA was ordered from RIKEN (RAFL15-04-H19) and amplified using the following primers: FATG, 5´- ATGTTTTCTTGCATAGCTTGTAC and Rstop, 5´-TTAGAGGTACTGTGTTTGTATTC. The PCR product was cloned into the pCR8/GW/TOPO entry vector (Invitrogen) and recombined by LR reaction into the

pMDC32 destination vector (Curtis and Grossniklaus, 2003). The *pMDC32-35S:BRX* construct was transferred to Agrobacterium tumefaciens C58C1 (pGV2260; Deblaere et al., 1985) by electroporation and used to transform the *brx-2* mutant (phosphinothricin resistant) by the floral dipping method. Seeds of transformed plants were harvested and plated on hygromycin (20 μg/ml) selection medium to identify T1 transgenic plants. T3 progenies that were homozygous for the selection marker were used for further studies.

Germination and root growth assays

To determine sensitivity to inhibition of germination by ABA the medium was supplemented with 0.5 or 0.8 μM ABA. To score seed germination, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined. Approximately 200 seeds of each genotype were sowed in each medium and scored for germination and early growth 10 days later. For root growth assays, seedlings were grown on vertically oriented MS medium plates for 4 to 5 days. Afterwards, 20 plants were transferred to new plates containing MS medium lacking or supplemented with the indicated concentrations of ABA or brassinolide. After the indicated period of time, the plates were scanned on a flatbed scanner to produce image files suitable for quantitative analysis using the NIH Image software (ImageJ v1.37).

RNA analysis

Root tissue was collected from two-week old plants that were either mock- or 10 μM ABA-treated for 3 h and frozen in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy Plant Mini Kit and 1 μg of the RNA solution obtained was reverse transcribed using 0.1 μg oligo$(dT)_{15}$ primer and M-MLV reverse transcriptase (Roche), to finally obtain a 40 μl cDNA solution. RT-qPCR amplifications and measurements were performed using an ABI PRISM 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems). The sequences of the primers used for PCR amplifications were the following ones: for *BRX*, forward 5´- AGTCAGATTCAGCCGGGAACG and Rstop, for *HAB1* (At1g72770), forward 5´-AACTGCTGTTGTTGCCTTG and reverse 5´-GGTTCTGGT CTTGAACTTTCT; for *ABI1* (At4g26080), forward 5´-ATGATCAGCAG AACAGAGAGT and reverse 5´-TCAGTTCAAGGGTTTGCT; for *ABI2* (At5g57050) forward 5´-AGTGAC TTCAGTGCGGCGAGT and reverse 5´- CCTTCTTTTTC AATTCAAGGAT; for *PP2CA* (At3g11410), forward 5´-CTTTGTCGTAACGGTGTAGC

and reverse 5´-TTGCTCTAGACA TGGCAAGA, and for *β-actin-8* (At1g49420), forward 5´-AGTGGTCGT ACAACCGGTATTGT and reverse 5´-GAGGATAGCATGTGGAAG TGAGAA.

RT-qPCR amplifications were monitored using the Eva-Green™ fluorescent stain (Biotium). Relative quantification of gene expression data was carried out using the $2^{-\Delta\Delta C_T}$ or comparative C_T method (Livak and Schmittgen 2001). Expression levels were normalized using the C_T values obtained for the *β-actin-8* gene. The presence of a single PCR product was further verified by dissociation analysis in all amplifications. All quantifications were made in triplicate on RNA samples obtained from three independent biological replicates.

RNA amplification and labeling for microarray analysis

Total RNA (1.25 μg) from three independent biological replicates was amplified and amino allyl-labeled using MessageAmp® II aRNA kit (Ambion, http://www.ambion.com) and 5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate (aa-dUTP, Ambion), according to manufacturer's instructions. Approximately 80-90 µg of amplified amino allyl RNA (aRNA) was obtained. For each sample, 7.5 µg of aRNA was resuspended in the coupling buffer and labeled with either Cy3 or Cy5 Mono NHS Ester (Cy™ Dye Post-labelling Reactive Dye Pack, Amersham). The samples were purified with Megaclear™ (Ambion) according to manufacturer's instructions. Incorporation of Cy3 and Cy5 was measured using 1 µl of the probe in a Nanodrop spectrophotometer (Nanodrop Technologies Inc.; http://www.nanodrop.com/). For each hybridization 200 pmol of Cy3 and Cy5 probes was mixed and volume reduced to 5 μl in a speed-vac. 20 μg Poly(A) and 20 μg of yeast tRNA (Sigma Aldrich) were added. Each mixed probe was fragmented by adding 1 µl of 10X fragmentation buffer (Ambion) and incubating at 70°C for 15 min. The reaction was stopped with 1 µl of stop solution (Ambion). The 11 μl final volume of each mixed probe was diluted in 90 μl of hybridization solution.

Microarray hybridization

Three biological replicates were independently hybridized for each transcriptomic comparison. Microarray slides were composed of synthetic 70 mer oligonucleotides from the Operon *Arabidopsis* Genome Oligo Set Version 3.0 (Qiagen; http://www.qiagen.com/) spotted on aminosilane-coated slides (Telechem, http://www.arrayit.com) by the University of Arizona. Slides were rehydrated and UV-crosslinked according to the details on the supplier's web page http://ag.arizona.edu/microarray/ methods.html. The slides were then washed twice for 2 min in 0.1% SDS, in sterile water for 30 sec and dipped in ethanol for 3 minutes with shaking. Arrays were drained with a 2000 g spin for 10 min. Slides were pre-hybridized in 6X SSC (Sigma), 0.5% SDS (w/v) (Sigma), and 1% BSA (w/v) at 42°C for 1 h., followed by 2 washes with milliQ water for 1 minute and one rinse with isopropanol. Excess water was drained with a 2000 g spin for 10 min. For the hybridization, equal amounts of dye of each aRNA labeled with either cy3 or cy5, ranging from 200 to 300 pmol, were mixed with 20 µg of polyA and 20 µg of yeast tRNA (Sigma-Aldrich) in a volume of 9 µl. To this volume 1 µl of RNA fragmentation buffer was added, (RNA Fragmentation Reagents, Ambion) and, after 15 min at 70°C, 1 µl of stop solution. Fragmented labeled RNA was directly mixed with hybridization solution containing 50 µl deionized formamide (Sigma), 30 µl 20 × SSC, 5 µl 100 × Denhardt's solution (Sigma) and 5 µl 10% SDS in a final volume of 100 µl. The hybridization mixture was denatured at 95°C for 5 min, spun briefly and applied by capillary between a pre-treated slide (see above) and a 60 × 42 mm coverslip LifterSlip (Erie Scientific). Slides were incubated overnight at 42°C in a microarray hybridization chamber (ArrayIt Hybridization Cassette, TeleChem). The next morning, slides were washed sequentially once in 1 × SSC 0.1% SDS 5 min at 30°C; once in 0.2 × SSC 0.1% SDS 5 min at 30°C; twice in 0.1 × SSC 2 min each at 30°C; and finally 6 times at 0.01 × SSC for 2 min at 25°C. Slides were dried by centrifugation at 2000g for 10 min at room temperature. Hybridized microarray slides were scanned right after at 532 nm for Cy3 and 635 nm for Cy5, with a GenePix 4000B scanner (Axon Molecular Devices,http://www.moleculardevices.com), at 10 nm resolution and 100% laser power. Photomultiplier tube voltages were adjusted manually to equal the overall signal intensity for each channel, to increase the signal- to-noise ratio, and to reduce the number of spots with saturated pixels. Spot intensities were quantified using genepix pro 6.0 microarray-analysis software (Axon Molecular Devices). Data were normalized

by mean global intensity and with lowess (locally weighted scatter plot) correction (Yang *et al.*, 2001) using Genepix pro 6.0 and Acuity 4.0 software (Axon Molecular Devices), respectively. After image analysis, spots with a net intensity in both channels lower than twice the median signal background were removed as low-signal spots, and only probes for which we obtained valid data at least in the two of the three slides were considered for further analysis.

Identification of differentially expressed genes and gene ontology analysis

Significance Analysis of Microarrays, SAM, (Tusher et al., 2001) was performed on the three normalized data sets to identify differentially expressed genes. The parameters for SAM were adjusted so that the false discovery rate (FDR) for every experiment was 0.05. A 2 fold expression cut-off was considered to determine up-regulated and down-regulated genes. In order to establish differences in expression between the two genotypes, a 1.4 fold threshold and a FDR of 0.05 was considered. A functional category analysis (FunCat) analysis of the genes simultaneously up-regulated or down-regulated in the two genotypes was carried out by MIPS (http://mips.gsf.de/proj/funcatDB/search_main_frame.html). Only over represented categories with a p-value smaller than 0.05 were further considered. Venn diagrams were generated to illustrate differences in expression.

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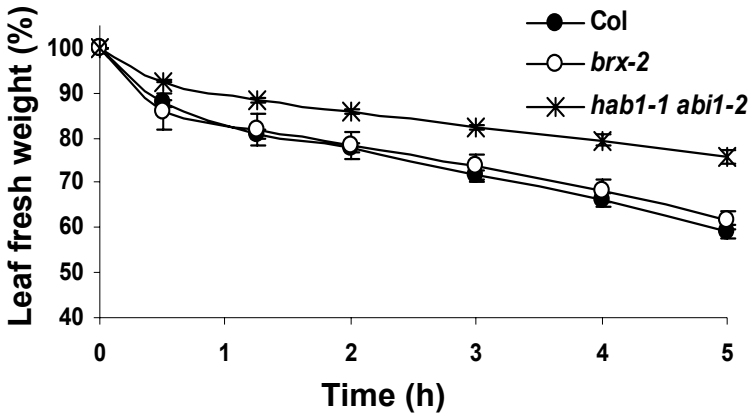
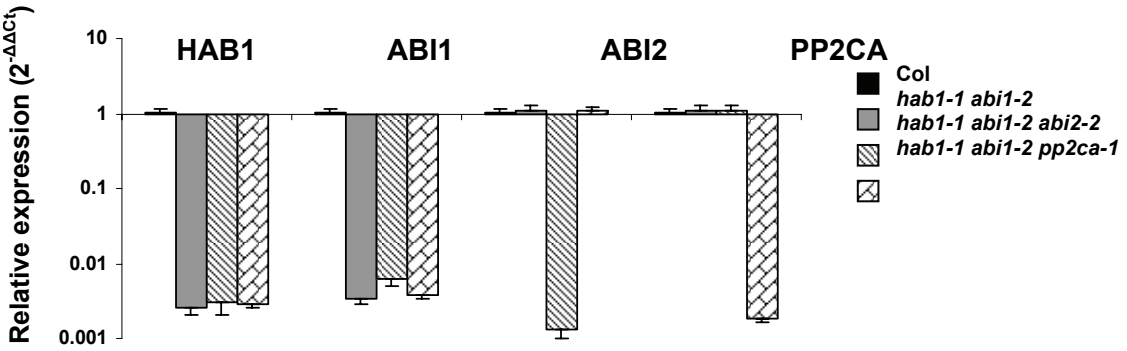
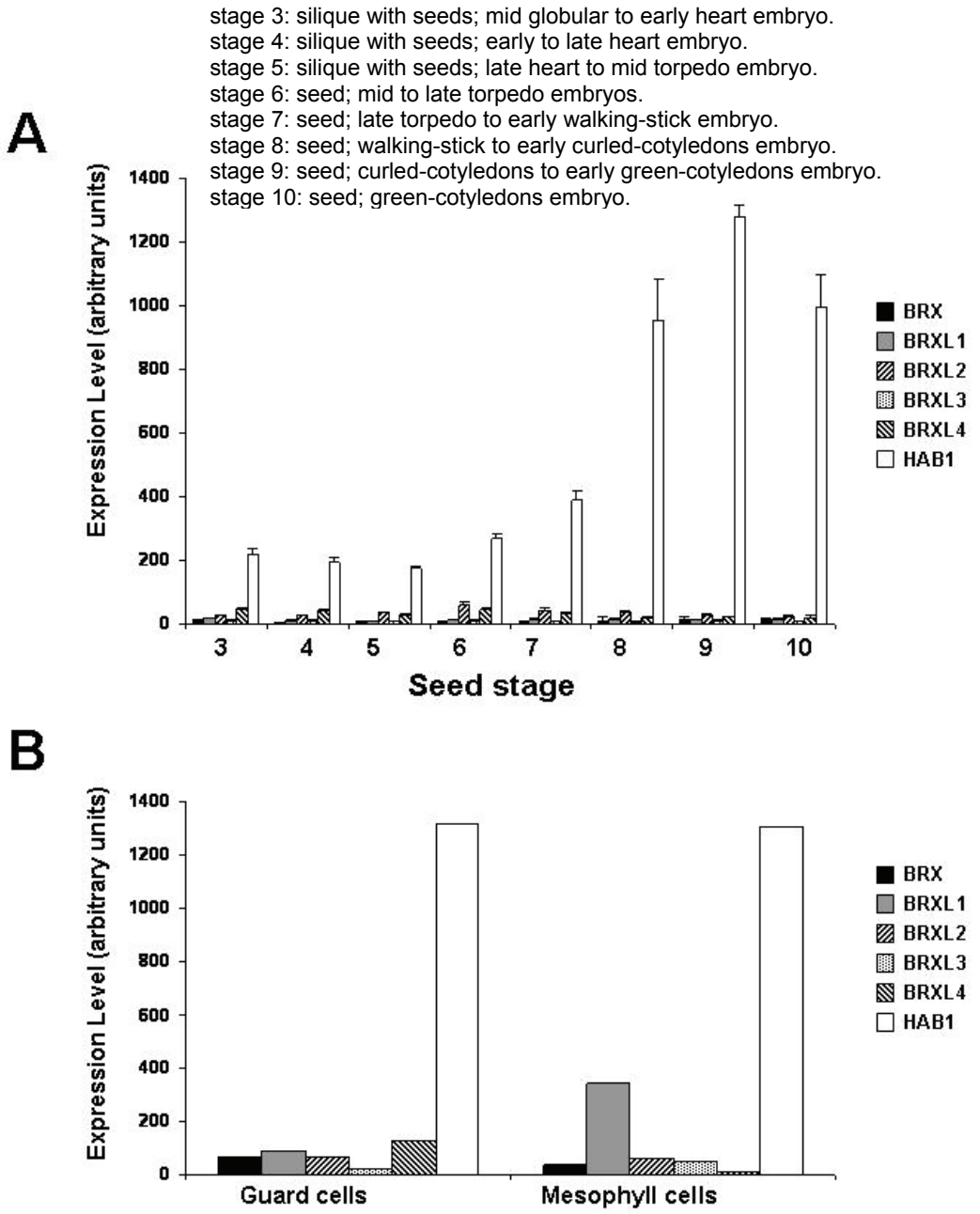


Supplemental Figure S1. Detached-leaves water-loss assays show similar water-loss in *brx-2* and wild type, whereas reduced water-loss was found in the double *hab1-1abi1-2* mutant.



Supplemental Figure S2. RT-qPCR analysis of *HAB1*, *ABI1*, *ABI2* and *PP2CA* expression in wild type, double *hab1-1abi1-2* and triple *pp2c* ko mRNAs prepared from 2-week-old roots.



Supplemental Figure S3. Expression levels of *BRX*, *BRX-like* (*BRXL*) and *HAB1* genes at different stages of embryo development and guard/mesophyll cells.

3. Putative components of SWI/SNIF complexes play an important role in ABA signaling

Part of the work presented in this chapter has been published:
Saez A*, Rodrigues A*, Santiago J, Rubio S, Rodriguez PL.(2008). HAB1-SWI3B Interaction Reveals a Link between Abscisic Acid Signaling and Putative SWI/SNF Chromatin-Remodeling Complexes in Arabidopsis. Plant Cell 20(11): 20: 2972-2988.
(*)These authors contributed equally to this work.
The PDF version of this paper is presented in supplement

Abstract

Abscisic acid (ABA) has an important role for plant growth, development and stress adaptation. HAB1 is a protein phosphatase type-2C (PP2C) that plays a key role as negative regulator of ABA signaling; however, the molecular details of HAB1 action in this process are not known. A two hybrid screening revealed that AtSWI3B, an *Arabidopsis* homolog of the yeast SWI3 subunit of SWI/SNF chromatin remodeling complexes, is a prevalent interacting partner of HAB1. The interaction mapped to the N-terminal half of AtSWI3B and required an intact protein phosphatase catalytic domain. Bimolecular fluorescence complementation (BiFC) and coimmunoprecipitation assays confirmed the interaction of HAB1 and SWI3B in the nucleus of plant cells. Yeast two hybrid and BiFC also confirmed the interaction of SWI3B with ABI1, ABI2 and PP2CA, though this interaction was weaker than the observed with HAB1. *swi3b* mutants showed a reduced sensitivity to ABA-mediated inhibition of seed germination and growth, and reduced expression of the ABA-responsive genes *RAB18* and *RD29B*. Chromatin immunoprecipitation (ChIP) experiments showed that the presence of HAB1 in the vicinity of *RD29B* and *RAB18* promoters was abolished by ABA, which suggests a direct involvement of HAB1 in regulation of ABA-induced transcription. Contrary to the phenotypes presented by *swi3b* mutants, mutants of BRM and SYD, other putative members of Arabidopsis SWI/SNF complex, showed hypersensitivity to ABA in germination, root growth and enhanced expression of ABA responsive genes. Additionally, our results uncover AtSWI3B as a novel positive regulator of ABA signaling and suggest that HAB1 modulates ABA response through regulation of a putative SWI/SNF chromatin remodeling complex.

Introduction

The phytohormone abscisic acid (ABA) is a key regulator of plant growth and development as well as plant responses to situations of decreased water availability. A fast mechanism to adjust ABA levels and respond to changing environmental cues is the hydrolysis of glucose-conjugated ABA (Lee et al., 2006). Additionally, water stress leads to the accumulation of ABA through enhanced expression of ABA biosynthetic genes, mainly *9-cis-epoxycarotenoid dioxygenase 3* (Nambara and Marion-Poll, 2005; Barrero et al., 2006). ABA triggers a variety of adaptive responses, such as stomatal closure and differential gene expression, which are crucial for plant survival under stress conditions (Schroeder et al., 2001; Nambara and Marion-Poll, 2005).

Decades of research in ABA signaling have resulted in the identification of many elements of the ABA signal transduction pathway, including both negative and positive regulators (reviewed by Finkelstein et al., 2002; Himmelbach et al., 2003; Israelsson et al., 2006). Under water stress, ABA signaling leads to coordinated remodeling of gene expression, which affects more than ~5% of plant transcriptome (Huang et al., 2007). Downstream nuclear effects of ABA are mediated by different transcription factors (TFs) that play a positive role in ABA signaling, which comprise ABA-responsive element (ABRE)-binding proteins (ABI5/ABF/AREB/AtbZIP family) (Finkelstein and Lynch, 2000; Uno et al., 2000; Choi et al., 2000; Bensmihen et al., 2002); *Arabidopsis* ABI3 and maize (*Zea mays*) VP1 TFs of the B3 domain family (McCarty et al., 1991, Giraudat et al., 1992), the ABI4 TF from the APETALA2 (AP2) domain family (Finkelstein et al., 1998), ATMYC2 and ATMYB2 TFs (Abe et al., 2003). Some TFs that function as transcriptional repressors of ABA response have been also described (Himmelbach et al., 2002; Song et al., 2005; Pandey et al., 2005). In eukaryotes, the packaging of DNA into chromatin implies that both transcriptional activators and repressors work together with large multi-subunit complexes that remodel nucleosomes to regulate gene expression (Carrozza et al., 2003; Smith and Peterson, 2005). Two general classes of chromatin-modifying factors can be distinguished, those that covalently modify the amino-terminal tails of histone proteins and those that utilize ATP hydrolysis to remodel or reposition nucleosomes (Carrozza et al., 2003; Smith and Peterson, 2005). The first class includes protein complexes that acetylate or deacetylate lysine residues present in the amino termini of histone proteins (histone acetyltransferases, HATs) and histone deacetylases (HDACs). The second class of factors is composed of ATP-dependent chromatin remodeling complexes, which alter nucleosome structure or positioning. Among them, the yeast SWI/SNF complex was the first one to be described (Cairns et al., 1994; Peterson et al., 1994). Arabidopsis has four SWI/SNF ATPases: SPLAYED (SYD) and BRAHMA (BRM), CHR12 and CHR23 (Flaus et al., 2006). All four have the same domains in their N-terminal sequence. Only BRM has a C-terminal bromodomain. The molecular properties of the large and unstructured C-terminal sequence of SYD are not well understood (Knizewski et al., 2008).

In addition to the ATPase Swi2/Snf2, it contains a central core composed by three additional polypeptides, i.e Swi3, Snf5 and Swp73, which are required for the assembly and activity of the complex (Cairns and Kingston, 2000; Smith and Peterson, 2005; Yang et al., 2007).

Some reports of chromatin modifying factors that affect ABA response have been described (Song et al., 2005; Sridha and Wu, 2006), however, taking into account the deep impact of ABA on the regulation of gene expression and the many TFs involved in this process, we can envisage that many elements in this field are yet to be discovered.

PP2Cs were identified as key components of ABA signaling from pioneer work with the ABA-insensitive *abi1-1* and *abi2-1* mutants (Koornneef et al., 1984; Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997; Rodriguez, et al., 1998b). Currently, at least six *Arabidopsis* PP2Cs, namely ABI1, ABI2, PP2CA/AHG3, AHG1, HAB1 and HAB2, are known to regulate ABA signaling. Genetic approaches indicate that these PP2Cs are negative regulators of ABA signaling (Gosti et al., 1999; Merlot et al., 2001; Tahtiharju and Palva, 2001; Gonzalez-Garcia et al., 2003; Saez et al., 2004; Leonhardt et al., 2004; Yoshida et al., 2006b; Kuhn, et al., 2006; Saez et al., 2006; Nishimura et al., 2007). Although interacting partners for some of these PP2Cs have been described (Cherel et al., 2002; Himmelbach et al., 2002; Guo et al., 2002; Ohta et al., 2003; Yoshida et al., 2006a; Yang et al., 2006; Miao et al., 2006), the overall knowledge on their targets and their role in ABA signaling is far from complete. In this work, we have pursued a two-hybrid approach using the PP2C HAB1 as bait to identify putative interacting preys. Interestingly, a prevalent interacting partner of HAB1 was found to be the AtSWI3B protein, which is an *Arabidopsis* homolog of the SWI3 core subunit of SWI/SNF chromatin remodeling complexes (Sarnowski et al., 2002; Zhou et al., 2003). These complexes, already characterized in yeast, *Drosophila* and mammals, have not been yet biochemically characterized in plants, although genome analysis suggests that *Arabidopsis* contains the active components required to form such complexes (Farrona et al., 2004; Sarnowski et al., 2005). Thus, four SWI3-like proteins, i.e. SWI3A, SWI3B, SWI3C and SWI3D, have been identified (Sarnowski et al., 2002; Zhou et al., 2003) as well as other putative components of SWI/SNF complexes (Brzeski et al., 1999; Farrona et al., 2004; Bezhani et al., 2007). Current data on loci that encode putative components of SWI/SNF chromatin-remodeling complexes show that they operate as modifiers of transcriptional or epigenetic regulation in plant growth and development (Kwon and Wagner, 2007). Our data provide a link between a component of the ABA signaling pathway and a putative component of SWI/SNF chromatin-remodeling complexes, and therefore suggest that these complexes are also involved in hormonal response to abiotic stress.

Results

Previous results obtained at our laboratory: A yeast two hybrid screen revealed that AtSWI3B, an Arabidopsis homolog of the yeast SWI3 subunit of SWI/SNF chromatin remodeling complexes, is a prevalent interacting partner of HAB1. In contrast with SWI3B, none of the SWI3A, SWI3C and SWI3D proteins interact with HAB1. The interaction mapped to first 220 amino acid residues of AtSWI3B and required an intact protein phosphatase catalytic domain. Bimolecular fluorescence complementation (BiFC) and coimmunoprecipitation assays confirmed the interaction of HAB1 and SWI3B in the nucleus of plant cells. Chromatin immunoprecipitation (ChIP) experiments showed that the presence of HAB1 in the vicinity of RD29B and RAB18 promoters was abolished by ABA, which suggests a direct involvement of HAB1 in regulation of ABA-induced transcription. Analysis of ABA responsiveness in the progeny of *atswi3b* +/- hemizygous seedlings, which represents a ~2:1 mixture of hemizygous and wild type seeds (the homozygous are embryo lethal) revealed a reduced sensitivity to ABA of *atswi3b* +/- seeds and seedlings compared to wild type.

SWI3B interacts with ABI1, ABI2 and PP2CA

HAB1 belongs to a group of PP2Cs (clade A, Schweighofer et al., 2004) where six of the identified genes are associated with ABA signaling. Gene expression data and genetic analysis indicate that HAB1, PP2CA, ABI1 and ABI2 play a predominant role in ABA signaling both in seeds and vegetative tissue (http://www.genevestigator.ethz.ch; Saez et al., 2004 and 2006; Kuhn, et al., 2006). Therefore, we generated N-terminal truncations of PP2CA, ABI1 and ABI2 fused to GBD and their interaction with SWI3B was examined (Figure 1A). ΔNPP2CA, ΔNABI1 and ΔNABI2 were able to interact with SWI3B, although it was apparent in the growth assay that the interaction was weaker than that observed for ΔNHAB1. All fusion proteins were expressed at similar levels as verified by protein gel blot analysis using antibodies against GAD and GBD.

Bimolecular fluorescence complementation (BiFC) assays were used to detect the interaction between ABI1, ABI2 and PP2C with AtSWI3B in plant cells. To this end, ABI1, ABI2 and PP2C were fused to the N-terminal 155-amino acid portion of yellow fluorescent protein (YFP^N) in the pYFP^{N}43 vector. On the other hand, the N-terminal half of SWI3B was translationally fused to the C-terminal 84-amino acid portion of yellow fluorescent protein (YFP^C) in the pSPYCE vector, which generated a SWI3B-epitope HA-YFP^c fusion protein. The corresponding constructs were co-delivered into leaf cells of tobacco by *Agrobacterium* infiltration and as a result, fluorescence was observed in the nucleus of tobacco cells (Figure 1B). No fluorescence signal was observed when YFP^N43-*ABI1*, YFP^N43-*ABI2* or YFP^N43-*PP2CA* vector was co-delivered with pSPYCE or when pSPYCE-*SWI3B* was co-delivered with YFP^N43.

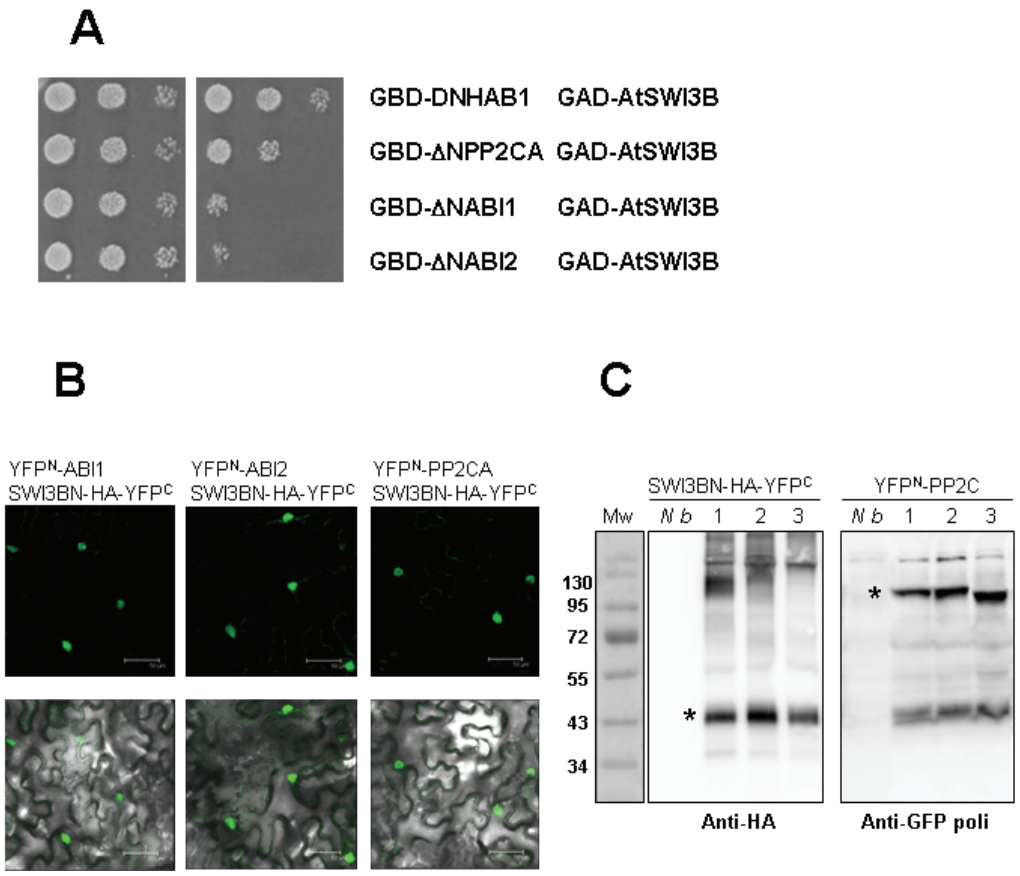


Figure 1. SWI3B interacts with ABI1, ABI2 and PP2CA. **(A)** Yeast two-hybrid assay. Interaction was determined by growth assay on media lacking His and Ade. Dilutions (10^{-1}, 10^{-2}, 10^{-3}) of saturated cultures were spotted onto the plates. Interaction assay with ΔNHAB1, ΔNPP2CA, ΔNABI1 and ΔNABI2 as baits, and SWI3B as prey. **(B)** BiFC assays show interaction of ABI1, ABI2 and PP2CA with SWI3B in the nucleus of tobacco leaves. Cells were infiltrated with a mixture of *Agrobacterium* suspensions harboring constructs SWI3BN-HA-YFP^C/ YFP^N-ABI1 (panel 3), /YFP^N-ABI2 (panel 4), /YFP^N-PP2CA (panel 5) and the silencing suppressor p19. **(C)** Protein gel blot analysis demonstrates expression of SWI3BN-HA-YFPC and the corresponding YFPN-PP2Cs (marked with asterisks). Protein extracts obtained from tobacco leaves infiltrated with Agrobacterium suspensions harboring the silencing suppressor p19 and constructs SWI3BN-HA-YFPC/ YFPN-ABI1 (lane 3), /YFPN-ABI2 (lane 4) and /YFPN-PP2CA (lane 5), respectively, or p19 alone (p19) were analysed using anti-HA or anti-GFPN antibodies.

Expression of fusion proteins was verified by protein gel blot analysis using antibodies against the epitope HA and peptide comprising amino acids 3-17 of GFP (anti-GFP^N) (Figure 1C). .

Selection of targeting induced local lesions of SWI3B in the Arabidopsis genome: *swi3b* Tilling mutants

Knockout swi3b mutants are embryo lethal (Sarnowski et al., 2005). To better understand SWI3B function we searched for viable mutants by using a

TILLING approach in *Arabidopsis* (Col-*er105* background) (Till et al, 2003) (http://tilling.fhcrc.org:9366/home.html). The SANT (for SWI3-ADA2-NCoR-TFIIIB) domain (amino acids 224 to 272) is not necessary for the interaction with HAB1, as indicated by the two hybrid results, but is probably involved in crucial protein-protein interactions (Mohrmann and Verrijzer, 2005) as for instance the interaction with the SWI2/SNF2 ATPase (Hurtado et al., 2006). Therefore, we decided to focus in point mutations in this domain. Thirteen mutants were recovered by the *Arabidopsis* TILLING Program. According to SIFT (Sorting Intolerant From Tolerant) (sift score <0.05) (Ng and Henikoff, 2001) and simultaneously to PSSM (Position Specific Scoring Matrix) (PSSM difference >10) (Henikoff et al., 1990) software analysis, 3 out of the 13 mutations were predicted to affect protein function (Table 1).

#	Nucleotide Change	Effect	PSSM difference[(a)]	SIFT Score[(b)]	Zygosity
1	G361A	L89=			Hetero
2	G830A	R217Q	**11.7**	0.06	Hetero
3	C866T	S229F	**11.7**	**0.01**	Homo
4	C886T	L236=			Hetero
5	G892A	E238K	**14.1**	**0.00**	Hetero
6	G911A	G244E	5	0.66	Hetero
7	G913A	D245N	**11.6**	**0.03**	Homo
8	C971T	S264F	8.6	**0.03**	Homo
9	G1050A	Q290=			Hetero
10	C1061T	S294F		0.31	Homo
11	C1132T	L318F	9.2	0.26	Hetero
12	G1228A	Intron			Hetero
13	G1344A	Intron			Homo

Table 1. Mutants recovered by the Arabidopsis TILLING Program and prediction of functional impact of amino-acid substitutions by sequence-homology based software tools. PSSM difference or SIFT Scores in bold indicate that the mutation is predicted to damage the protein
(a) Lower SIFT scores predict an amino acid change less likely to be tolerated.
(b) Higher PSSM differences predict an amino acid change less likely to be tolerated.

In order to avoid a preliminary step of selection, we decide to use only homozygous mutants. So, for phenotypical analysis we select the two homozygous mutant lines less likely to be tolerated (S229F and D245N), according to SIFT and PSSM analysis, and another one (S264F) that initially was not considered due to its PSSM difference lower than 10. However the *swi3bS229F* mutant presented seed germination defects and therefore it was not considered for further analysis. The

swi3bD245N and *swi3bS264F* alleles were named *swi3b-3* and *swi3b-4*, respectively (Figure 2A).

swi3b mutants show a reduced sensitivity to ABA-mediated inhibition of germination and growth as well as reduced expression of RD29B and RAB18

The *swi3b-1* and *swi3b-2* knockout mutants (Figure 2A) were previously reported to be embryo lethal (Sarnowski et al., 2005). Therefore, the progeny from *swi3b-1* and *swi3b-2* hemizygous plants was analysed to score ABA-mediated inhibition of germination and growth. These assays revealed a reduced sensitivity to ABA of *atswi3b* +/- seeds and seedlings compared to wild type suggesting that *AtSWI3B* is a positive regulator of ABA signalling ((data not showed).

Further evidence on the role of SWI3B in ABA signaling was obtained through the analysis of point mutations in *swi3b* alleles. Analysis of ABA-mediated inhibition of germination in *swi3b-3* and *swi3b-4* revealed that both mutants showed a reduced sensitivity to ABA in this assay compared to the Col-*er105* background (where TILLING mutants were originated) (Figure 2B). In particular, the *swi3b-3* mutant also showed a reduced sensitivity to ABA-mediated inhibition of early growth (Figure 2C). These results, together with those of *swi3b-1* and *swi3b-2* +/- seedlings, show that AtSWI3B is a positive regulator of ABA signaling that mediates ABA response in seeds and vegetative tissue. Additionally, we wondered whether SWI3B might play a role in regulation of gene expression in response to ABA. SWI3B is a putative core component of SWI/SNF complexes and chromatin remodelers have a well-established role in transcriptional regulation. Therefore, real-time quantitative polymerase chain reaction (RT-qPCR) was used to analyze the expression of the ABA-responsive *RD29B, RAB18, KIN1, RD22, RD29A* and *P5CS1* genes in wild type and *swi3b-3* mutant (Figure 2D). In general terms, these gene markers show a low expression in the absence of ABA or stress treatment, which is up-regulated in response to the inductive signal. Upon ABA treatment, expression of *RD29B* and *RAB18* in *swi3b-3* was 15% and 6%, respectively, of that found in wild type, whereas expression of the other gene markers did not differ more than 2-fold in both genotypes. Thus, SWI3B appears to regulate a subset of ABA-inducible genes, whereas its function seems to be partially dispensable or redundant for the expression of other ABA-responsive genes.

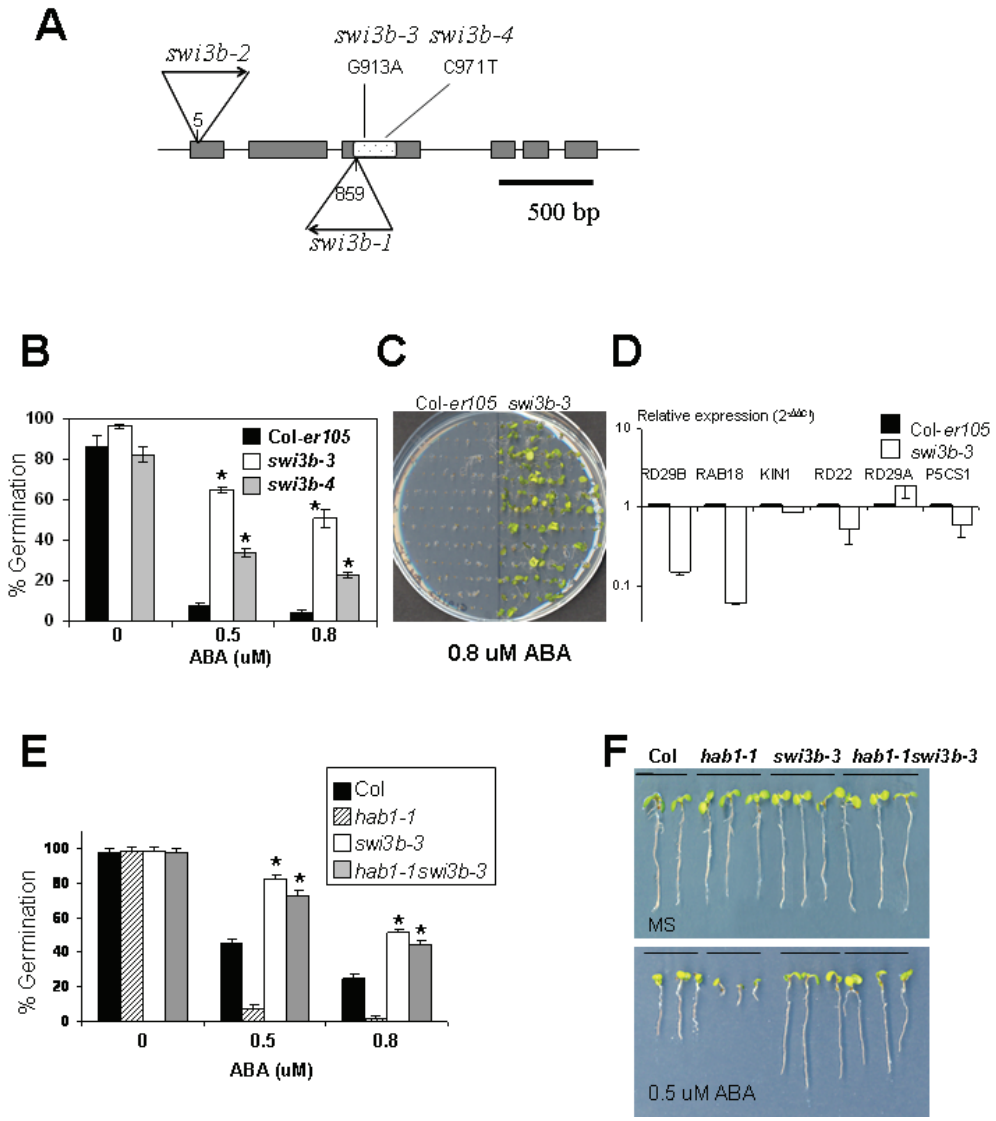


Figure 2. *swi3b* mutants show a reduced sensitivity to ABA-mediated inhibition of germination and growth. **(A)** T-DNA insertions in the *swi3b-1* and *swi3b-2* alleles and localization of EMS-induced mutations in *swi3b-3* and *swi3b-4* alleles. The numbering begins at the ATG translation start codon. The grey boxes represent exons. SANT domain is spotted within the third exon. **(B)** Reduced sensitivity to ABA-mediated inhibition of seed germination in *swi3b-3* and *swi3b-4* mutants compared to Col-*er105*. Percentage of seeds that showed radicle emergence 96 h after sowed, in the presence of the indicated concentrations of ABA. Values are averages ± SD for 3 independent experiments (n=200 seeds per experiment); * indicates P<0.01 (Student´s t test) when comparing data from each genotype and wt in the same assay conditions. **(C**) Reduced sensitivity to ABA-mediated inhibition of early growth in *swi3b-3* mutant compared to Col-*er105* in medium supplemented with 0.8 μM ABA. The photograph was taken at 18 days after sowing. **(D)** Reduced expression of ABA-inducible genes in *swi3b-3* compared to Col-*er105*. Values are the expression level reached in the mutant with respect to Col-*er105* (value 1) as determined by RT-qPCR analyses. Expression of gene markers was analysed in 7-day-old seedlings grown in medium supplemented with 0.3 μM ABA. **(E)** *swi3b-3* phenotype is epistatic to *hab1-1*. Percentage of seeds that showed radicle emergence 96 h after sowed. Values are averages ± SD for 3 independent experiments; * indicates P<0.01 (Student´s *t* test) when compared data from *swi3b-3* and wt, or *hab1-1swi3b-3* and *hab1-1*, in the same assay conditions. **(F)** Reduced sensitivity to ABA-mediated inhibition of early growth in *swi3b-3* and *hab1-1swi3b-3* double mutant. Photographs were taken at 7 (MS) and 11 (0.5 μM ABA) days after sowing. Plants were removed from growth medium and rearranged on plates for photography.

Finally, to further characterize the genetic relationship between the ABA-hypersensitive locus *hab1-1* and ABA-insensitive locus *swi3b-3*, we generated a *hab1-1swi3b-3* double mutant. Analysis of ABA-mediated inhibition of germination (Figure 2E) and early seedling growth (Figure 2F) revealed that *hab1-1swi3b-3* showed an ABA-insensitive phenotype, in contrast to *hab1-1*, which indicates that SWI3B is epistatic to HAB1, and therefore HAB1 functions upstream of SWI3B in the ABA signaling pathway. In addition to reduced sensitivity to ABA in the assays described above, the *swi3b-3* allele showed both impaired vegetative and reproductive growth (Supplemental figure 1), which likely reflects the capital role of SWI3B in plant growth and development as a core component of diverse SWI/SNF complexes (Zhou et al., 2003; Sarnowski et al., 2005; Bezhani et al., 2007). In agreement with this role, combination of the *swi3b-3* and *swi3b-2* alleles was embryo lethal (Sup. figure 1).

brm and _syd_ mutants show enhanced sensitivity to ABA-mediated inhibition of germination and growth as well as enhanced expression of RD29B and RAB18

SWI3B is a putative component of SWI/SNF chromatin remodelling complexes of Arabidopsis. So we wondered whether other putative elements of Arabidopsis SWI/SNF complexes, specially the core ATPAses, might influence ABA response. Therefore, we decide to examine ABA sensitivity of *brm-1*, *brm-3*, *syd-5*, *chr12* and *chr23* mutants in germination, root growth and water loss. *brm-1* and *brm-3* plants present pleiotropic phenotypes, stronger in *brm-1*, including defects in leaf morphology, root growth and flower development, and homozygous *brm-1* plants are completely sterile (Hurtado et al., 2006; Farrona., 2007). The phenotypic differences between *brm*-1 and *brm*-3 are probably related with the position of T-DNA insertions, in the first exon and 11th intron, respectively. *syd-5* plants are smaller than WT exhibit unique subtle cotyledon and leaf shape abnormalities and are sterile (Bezhani et al., 2007).

Analysis of ABA mediated inhibition of germination in *brm-1* and *brm-3* showed that both mutants present hypersensitivity to ABA in this assay compared with WT (figure 3B and 3C). Additionally both mutants also presented hypersensitivity to ABA-mediated root growth inhibition, more pronounced in the case of *brm-1* (figure 3D and 3E). However, water loss assays did not show significant differences between *brm* mutants and WT plants (figure 3F). We also wondered whether BRM may be involved in the regulation of gene expression in response to ABA. RT-qPCR was used to analyse de expression of the ABA responsive genes *RD29B* and *RAB18* in both *brm*

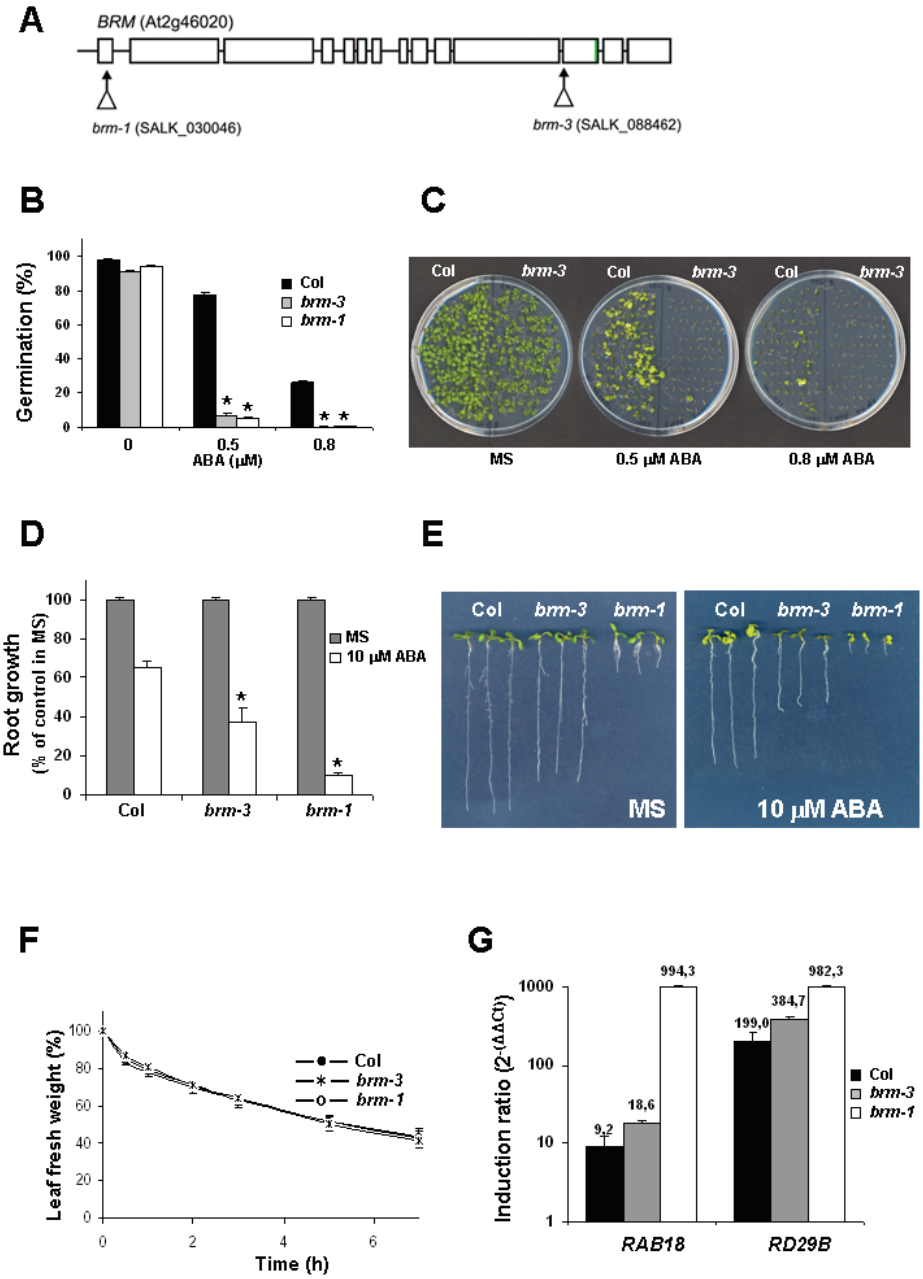


Figure 3. *brm* mutants show ABA-hypersensitive inhibition of germination and root growth (**A**) Structure of the *BRM* gene and location of T-DNA insertion sites of *brm* alleles. **(B)** Percent of seed that germinated and developed green cotyledons, in the presence of the indicated ABA concentration, after 11 days. Values are average ±SE of 3 independent experiments. * indicates P<0.01 (Student´s *t* test) when comparing data from each genotype and wt in the same assay conditions. **(C)** ABA hypersensitive germination and early growth of *brm-3* mutants. Photographs were taken at 11 (MS) and 18 (ABA) days after sowing. **(D)** ABA hypersensitive root growth inhibition of *brm* mutants. Values are average ±SE of 3 independent experiments. * indicates P<0.01 (Student´s *t* test) when comparing data from each genotype and wt in the same assay conditions. **(E)** Representative seedlings were selected and photographed after 5 days of the transfer of 4 days old seedlings to plates lacking or containing 10µM ABA. **(F)** Detached leaves water-loss assays. Four samples of three leaves of 21 days-old plants were excised and fresh weight was determined by submitting leaves to the drying atmosphere of a flow laminar hood for 7h. Measures are mean ±SE of 3 independent experiments. **(G)** Induction ratio (10 mM ABA/mock treatment) of *RAB18* and *RD29B* genes as measured by qRT-PCR. Values are average ±SE of 3 independent experiments.

mutants (figure 3G). *RD29B and RAB18* genes are more strongly upregulated in *brm* mutants, particularly in *brm-1*, than in WT. It is worth to notice that *brm-1* showed a stronger ABA hypersensitive phenotype than *brm-3*, which possibly reflects the expression in *brm-3* of a truncated BRM polypeptide lacking the last 454 residues (Farrona et al., 2007) as opposed to *brm-1*, in which no full length or truncated BRM proteins could be detected (Hurtado et al., 2006). The analysis of *syd-5* ABA response showed that this mutant is also ABA hypersensitive in germination (figure 4A) and root growth assays (figure 4C) and that *RD29B and RAB18* genes are more strongly upregulated in response to ABA than WT plants. Taking together, these results suggest that BRM and SYD are negative regulators of ABA signalling.

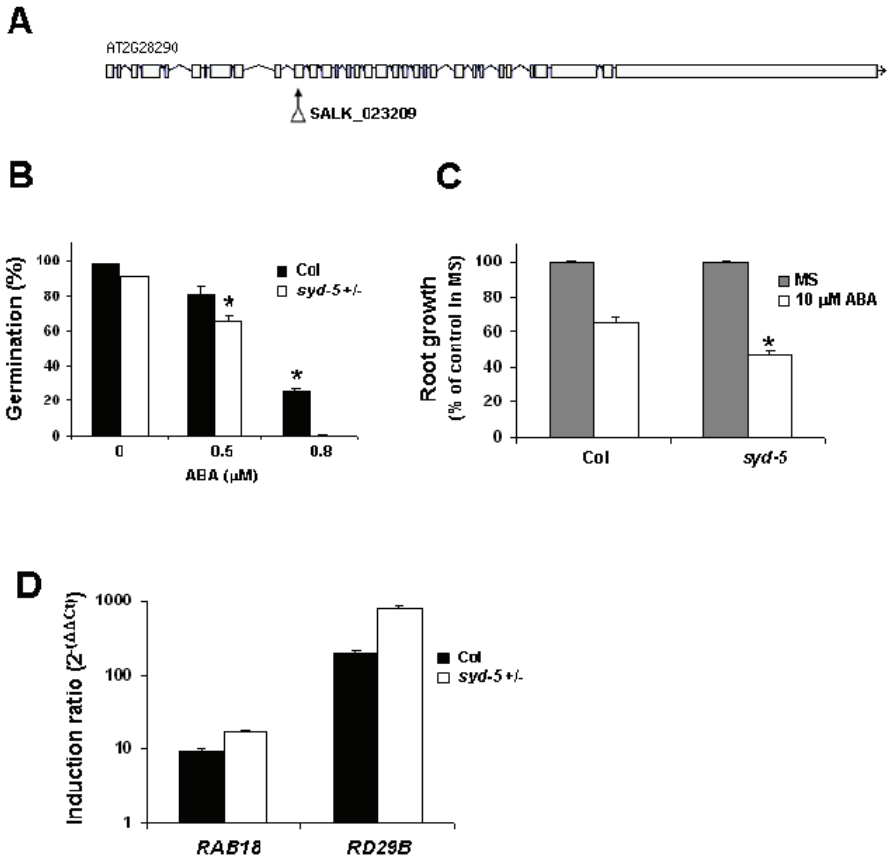


Figure 4. *syd* mutants show ABA-hypersensitive inhibition of germination and root growth **(A)** Structure of the SYD gene and location of T-DNA insertion site **(B)** Percent of seed that germinated and developed green cotyledons, in the presence of the indicated ABA concentration, after 11 days. Values are average ±SE of 3 experiments. * indicates P<0.01 (Student´s t *t*est) when comparing data from syd-5 +/- and wt in the same assay conditions. **(C)** ABA hypersensitive root growth inhibition of *syd-5* mutants. . Values are average ±SE of 3 independent experiments. * indicates P<0.01 (Student´s *t* test) when comparing data from syd-5 +/- and wt in the same assay conditions. **(D)** Induction ratio (10 mM ABA/mock treatment) of *RAB18* and *RD29B* genes as measured by qRT-PCR. Values are average ±SE of 3 independent experiments.

On contrary, CHR12 and CHR23 mutants showed a response to ABA similar to WT, in germination, root growth inhibition, water loss assays and gene expression (figure 5). Considering the very high level of similarity between CHR12 and CHR23 (supplemental figure 2) these results might reflect functional redundancy between these two SWI/SNF ATPases, that could also explain the absence of developmental defects on CHR12 and CHR23 single mutants, on contrary of what happens in BRM and SYD single mutants.

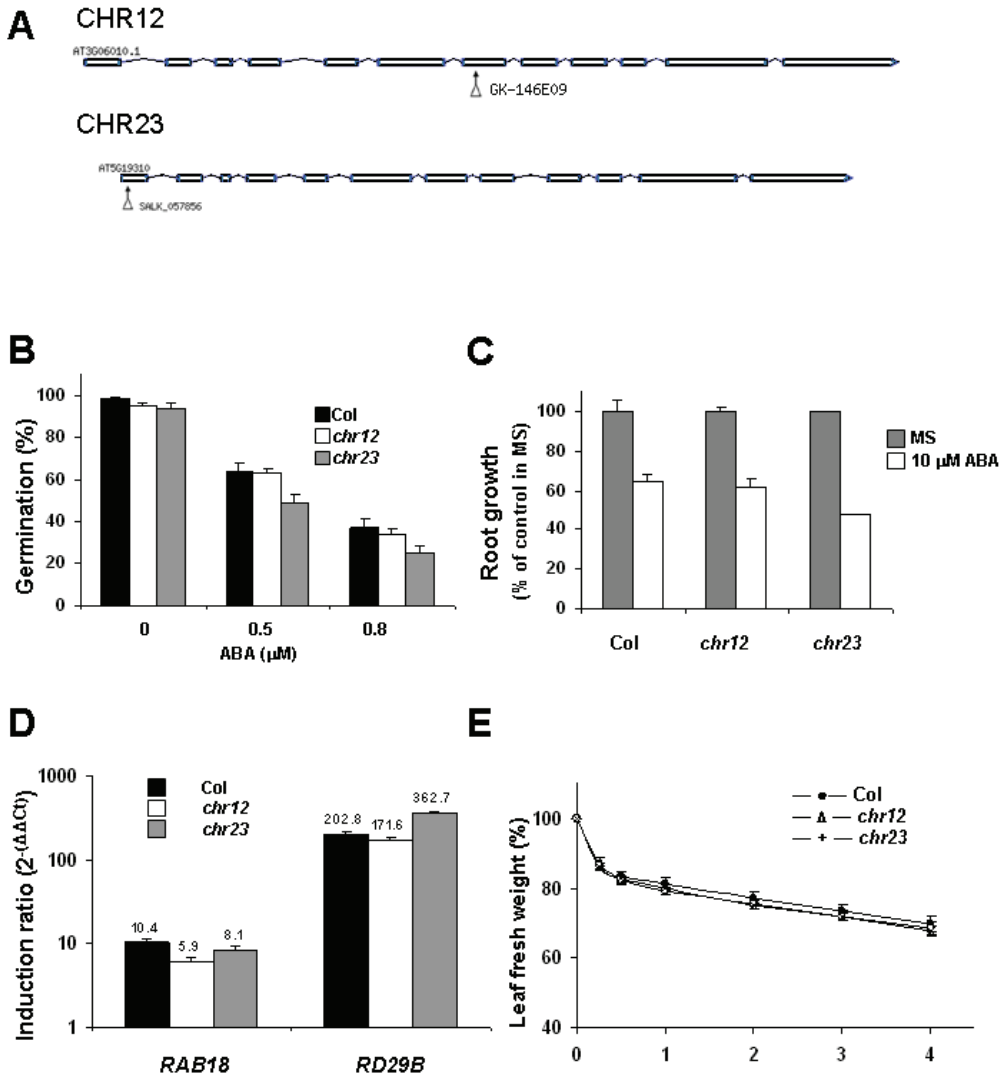


Figure 5. CHR12 and CHR23 mutants show an ABA response similar to WT. **(A)** Structure of the CHR12 and CHR23 genes and location of T-DNA insertion sites. **(B)** Percent of seed that germinated and developed green cotyledons, in the presence of the indicated ABA concentration, after 11 days. Values are average ±SE of 3 experiments. **(C)** ABA root growth inhibition assay. **(D)** Induction ratio (10 μM ABA/mock treatment) of RAB18 and RD29B genes as measured by qRT-PCR. Values are average ±SE of 3 independent experiments. **(E)** Detached leaves water-loss assays. Measures are mean ±SE of 3 independent experiments

Discussion

Both gain-of-function and loss-of-function phenotypes of the PP2C HAB1 are consistent with a role as negative regulator of ABA signaling (Saez et al., 2004; Leonhardt et al., 2004). Thus, whereas constitutive expression of *HAB1* (*35S:HAB1*) led to reduced ABA sensitivity both in seeds and vegetative tissues, the recessive *hab1-1* mutant showed ABA-hypersensitive inhibition of seed germination and growth, enhanced ABA-mediated stomatal closure and enhanced expression of ABA-responsive genes (Leonhardt et al., 2004; Saez et al., 2004 and 2006). The ABA-hypersensitive phenotype of *hab1-1* was strongly reinforced when combined with a loss-of-function allele of *ABI1* (Saez et al., 2006). A critical aspect to improve our knowledge on HAB1 function and its role in ABA signaling is the identification of its interacting partners.

Physical interaction of HAB1 and SWI3B

A two hybrid assay revealed a strong interaction between the HAB1 catalytic domain and *SWI3B*. Serial deletions of AtSWI3B mapped the interacting domain to the N-terminal half of the protein.

The HAB1 mutant allele G246D ΔNhab1, which had less than 3% in vitro PP2C activity than wild type, did not interact with AtSWI3B neither in the two-hybrid nor BiFC assays. The G246D substitution affects the catalytic centre of the PP2C, and according to the crystal structure of human PP2C (Das et al., 1996), such mutation is expected to disturb the metal-coordinating residues Asp243 and Gly244 with concomitant reduction in catalytic activity. An alternative possibility has been postulated by Robert et al., (2006), which suggested that hab1^{Gly246Asp} might show enhanced affinity for its substrate and therefore enhanced dephosphorylating capacity. However, using casein as substrate, the in vitro PP2C activity of hab1^{Gly246Asp} was severely reduced compared to wild type, as it was the case for G246D ΔNhab1. Additionally, the equivalent Gly180Asp abi1-1 or Gly168Asp abi2-1 mutant proteins did not show enhanced affinity (just the opposite) for their interacting partners ATHB6/OST1 or SOS2/Prefibrillin, respectively (Himmelbach et al., 2002; Yoshida et al., 2006a; Ohta et al., 2003; Yang et al., 2006). In all these cases, including the interaction of HAB1 and SWI3B, it appears that a functional catalytic PP2C is required for binding of the different targets.

The interaction of HAB1 and AtSWI3B was confirmed in planta through BiFC and coimmunoprecipitation assays. HAB1 is localized both in nucleus and cytosol, however the BiFC assay clearly identified AtSWI3B as a nuclear target of HAB1. Interestingly, most of the targets previously identified for clade A PP2Cs were not nuclear proteins (Cherel et al., 2002; Guo et al., 2002; Ohta et al., 2003; Yoshida et al., 2006b; Yang et al., 2006; Miao et al., 2006). However, in the case of ABI1, it is supposed that the interaction with the TF ATHB6 must be nuclear (Himmelbach et al., 2002). Additionally, recent results reveal a nuclear localization signal (NLS) at the very end of the C-terminal domain of ABI1 that is required for regulating ABA-dependent gene expression (Moes et al., 2008). Inspection of the C-terminal amino acid sequence of HAB1, ABI2 and PP2CA also reveals a similar short region enriched in basic amino acids (Supplemental Figure 3). Additionally, the sequences of HAB1 and ABI2 display a second region that contains two positively charged clusters separated by a short linker region (Supplemental Figure 3). The nuclear interaction of PP2CA, ABI1 and ABI2 with AtSWI3B found in BiFC assays might be physiologically relevant to regulate ABA signaling. However additional experiments, e.g. ChIP analysis, will be required to confirm the presence of these PP2Cs in plant chromatin and specifically in ABA-regulated promoters. Six different AtSWI3B-interacting proteins have been described, namely SWI3A, SWI3C, SWI3D, BRM, SYD and BSH, which are putative components of SWI/SNF complexes (Sarnowski et al., 2002 and 2005; Bezhani et al., 2007). Surprisingly, on contrary of what happens with *swi3b* mutants, *brm* and *syd* mutants present an ABA hypersensitive phenotype in germination and root growth, and enhanced upregulation of ABA responsive genes. These results might suggest that BRM and SYD might be negative regulators of ABA signalling, rising the possibility that the ABA positive regulator role of SWI3B could partially, or totally, depend on a hypothetical negative regulation of SWI/SNF ATPases.

Role of HAB1, SWI3B and a putative SWI/SNF complex in ABA signaling

No SWI/SNF complex has been biochemically purified in plants, although comparative genome analysis indicates that plants encode a remarkably high number of potential components of such complex (Sarnowski et al., 2005). In yeast, *Drosophila* and mammals it is well known that an important subset of highly inducible genes requires SWI/SNF complex as transcriptional activator (Mohrmann and Verrijzer, 2005). It has

been previously reported that *hab1-1* mutants show 2-fold higher expression of ABA-responsive genes than wild-type plants (Saez et al., 2006), whereas *35S:HAB1* plants show reduced expression of ABA-inducible genes (Saez et al., 2004), therefore HAB1 negatively regulates expression of these genes. HAB1 is localized both in nucleus and cytosol, and therefore it could influence ABA signaling at different steps. ChIP experiments reveal the presence of HAB1 in the vicinity of the ABA-responsive *RAB18* and *RD29B* promoters, and ABA-treatment eliminates HAB1 from these regions. These results, taken together with the negative effect of HAB1 on the expression of ABA-inducible genes, strongly suggest a direct regulatory effect of HAB1 on ABA-mediated transcriptional regulation. Thus, the presence of HAB1 in the vicinity of ABA-responsive promoters correlates with inhibition of their transcription under basal conditions, whereas ABA-mediated removal of HAB1 from these regions appears to be required for full induction of them. In this context, both the HAB1-SWI3B interaction and the impaired up-regulation by ABA of *RAB18* and *RD29B* in *swi3b-3*, suggest that HAB1 might regulate a putative SWI/SNF complex targeted to some ABA-responsive promoters. The phenotypes described in this work for *swi3b-1* and *swi3b-2* +/- seedlings as well as *swi3b-3* and *swi3b-4* mutants are consistent with AtSWI3B acting as a positive regulator of ABA signaling. Taking into account the opposed roles of HAB1 and AtSWI3B in this signaling pathway, it is reasonable to postulate that HAB1 negatively regulates SWI3B function, modulating its role as positive regulator of ABA signaling. Alternatively, SWI3B might anchor HAB1 to a putative SWI/SNF complex, where the phosphatase activity of HAB1 might dephosphorylate a component required for proper function of the chromatin remodeler. Taking into account that the presence of HAB1 in the vicinity of the ABA-responsive *RD29B* and *RAB18* promoters is abolished by ABA, we speculate that ABA must inhibit HAB1 function, which releases its inhibitory effect on a yet unknown SWI/SNF complex involved in transcriptional activation of ABA-responsive genes. The ABA-hypersensitive phenotype of *brm* and *syd* mutants, indicating a possible role as negative regulators of ABA signalling of BRM and SYD, might suggest the possibility that SWI3B negatively influence the activity of these ATPases. Finally, it will be an exciting challenge for the future understanding how the dynamic structure of the chromatin is modulated in response to ABA to regulate gene expression as well as the characterization of cell signaling events that lead to chromatin remodeling.

Methods

Plant material

Arabidopsis thaliana (ecotype Columbia) and *Nicotiana benthamiana* plants were routinely grown under greenhouse conditions in pots containing a 1:3 perlite-soil mixture. For in vitro culture, *Arabidopsis* seeds were surface sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5%) containing 0.05% Triton X-100 for 10 min, and finally, four washes with sterile distilled water. Stratification of the seeds was conducted during 3 days at 4ºC. Afterwards, seeds were sowed on Murashige-Skoog (MS) plates containing solid medium composed of MS basal salts and 1% sucrose, solidified with 1% agar and pH adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 22ºC under a 16 h light, 8 h dark photoperiod at 80-100 μE m^{-2} sec^{-1}.

The *swi3b-1* (Koncz_2208) and *swi3b-2* (GABI_302G08) alleles are T-DNA mutants in Col background. They were kindly provided by Dr. G. Rios and have been previously described by Sarnowski et al., (2005). TILLING mutants were obtained through the *Arabidopsis* TILLING project, which performed a high-throughput reverse genetic screening to identify EMS-induced mutations in Col-*er105* background (Till et al., 2003). As a result, two alleles were identified, *swi3b-3* and *swi3b-4*, which showed changes with SIFT score <0.05, and therefore were predicted to be deleterious to the gene product (Ng and Henikoff, 2001). These mutants were backcrossed once with Col-*er105* and F2 homozygous mutants were genotyped by PCR amplification and DNA sequencing using the primers: F1261 and R1560. In the case of *swi3b-3*, a second backcross was done with Col and F2 *swi3b-3* mutants lacking the *er105* mutation were selected. In order to generate the *hab1-1swi3b-3* double mutant, we transferred pollen of *swi3b-3* (Col background) to the stigmas of emasculated flowers of *hab1-1* (Col background). The resulting F2 individuals were genotyped by PCR for the presence of the double mutant.

Yeast two-hybrid assays

The HAB1 coding sequence was excised from a pSK-HAB1 construct (Rodriguez et al., 1998a) using an *Eco*RI-*Sal*I double digestion and subcloned into *Eco*RI-*Sal*I doubly digested pGBT9 to generate an in frame fusion with the GBD. To generate the HAB1 deletion, the HAB1 sequence encoding the catalytic PP2C region (amino acid residues

179-511, ΔNHAB1) was amplified by PCR and blunt-end cloned into the *Eco*RV site from pBluescript SK (Stratagene, USA). The ΔNHAB1-coding sequence was *Eco*RI-*Sal*I excised and subcloned into pGBT9. The pGBT9-*ΔNHAB1* bait was transformed into the yeast strain AH109 (BD Biosciences).

The PP2CA cDNA was obtained from ABRC (clone M76G17STM). The PP2CA sequence encoding the catalytic PP2C region (amino acid residues 90-399) was amplified using the primers: FDNPP2CA and RPP2CA. The PCR product was cloned into the pCR8/GW/TOPO entry vector (Invitrogen) and the ΔNPP2CA-coding sequence was *Eco*RI-*Sal*I excised and subcloned into pGBT9. The ABI1 and ABI2 cDNAs were kindly provided by Dr Erwin Grill and have been described previously (Meyer et al., 1994; Rodriguez et al, 1998b). The ABI1 sequence encoding the catalytic PP2C region (amino acid residues 122-433) was excised using an *Eco*RI-*Pst*I double digestion and subcloned into pGBKT7 to generate pGBKT7-*ΔNABI1*. The ABI2 sequence encoding the catalytic PP2C region (amino acid residues 96-423, ΔNHAB2) was excised using a *Sca*I-*Sal*I double digestion and subcloned into pGBT9 to generate pGBT9-*ΔNABI2*.

Construction of plasmids

pACT2-SWI3B-C1 was generated from the pACT2-SWI3B full-length cDNA recovered from the two-hybrid screening through *Xho*I digestion and subsequent religation. pACT2-SWI3B-C2, pACT2-SWI3B-N1, pACT2-SWIRM and pACT2-ZZ were generated through PCR-mediated amplification using the following primer pairs, respectively: FATG and R660; F661 and R1410; FATG and R420; F400 and R660. Constructs that express fusion proteins between the GAD and SWI3A, SWI3B, SWI3C or SWI3D in the centromeric vector pPC86 were kindly provided by Dr J.C. Reyes (CABIMER, Spain) and they have been described by Hurtado et al., (2006). Protein fusion between the GBD binding domain and ΔNHAB1 were generated in the multicopy vector pGBT9 for the yeast two-hybrid screening or the centromeric vector pDBLeu for targeted interaction assays with SWI3-like proteins. The G246D mutation was introduced into the pGBT9-*ΔNHAB1* construct through replacement of a *Bgl*II-*Eco*RV fragment of HAB1 with a PCR-mutagenized version (see below).

Constructs to investigate the subcellular localization of HAB1 and SWI3B were generated in Gateway-compatible vectors. To this end, the coding sequences of HAB1

and SWI3B were PCR-amplified using the following pair primers, respectively: FBamHI and Rno-stop; FATG and R1407no-stop. The PCR products were cloned into the pCR8/GW/TOPO entry vector (Invitrogen) and recombined by LR reaction into the pMDC83 destination vector (Curtis and Grossniklaus, 2003).

Constructs to investigate in planta interaction using BiFC assays were done in the pSPYNE-35S and pSPYCE-35S vectors (Walter et al., 2004) as well as gateway vector pYFP^{N}43 (kindly provided by A. Ferrando, Universidad de Valencia, Spain). The coding sequences of HAB1 and G246D hab1 were excised from pCR8/GW/TOPO constructs using a double digestion *Bam*HI-*Stu*I and subcloned into pSPYCE doubly digested *Bam*HI-*Sma*I. The N-terminal half of SWI3B was excised from a pSK-SWI3B construct using a double digestion *Bam*HI-*Dra*I and subcloned into pSPYNE and pSPYCE doubly digested *Bam*HI-*Sma*I. Constructs where the basic leucine zipper (bZIP) transcription factor bZIP63 is cloned in pSPYNE-35S and pSPYCE-35S were kindly provided by Dr. J Kudla (University of Münster, Germany). The coding sequences of ABI1, ABI2 and PP2CA were PCR-amplified and cloned into pCR8/GW/TOPO and recombined by LR reaction into the pYFP^{N}43 destination vector.

Transient protein expression in N. *benthamiana*

Experiments were performed basically as described by Voinnet et al., (2003). The different binary vectors described above were introduced into *Agrobacterium tumefaciens* C58C1 (pGV2260) (Deblaere et al., 1985) by electroporation and transformed cells were selected in LB plates supplemented with kanamycin (50 μg/ml). Then, they were grown in liquid LB medium to late exponential phase and cells were harvested by centrifugation and resuspended in 10 mM morpholinoethanesulphonic (MES) acid-KOH pH 5.6 containing 10 mM MgCl2 and 150 μM acetosyringone to an $OD_{600\ nm}$ of 1. These cells were mixed with an equal volume of *Agrobacterium* C58C1 (pCH32 35S:p19) expressing the silencing suppressor p19 of tomato bushy stunt virus (Voinnet et al., 2003) so that the final density of *Agrobacterium* solution was about 1. Bacteria were incubated for 3 h at room temperature and then injected into young fully expanded leaves of 4-week-old *N. benthamiana* plants. Leaves were examined after 3-4 days under a Leica TCS-SL confocal microscope and laser scanning confocal imaging system. Samples for immunoblot and immunoprecipitation assays were harvested, frozen in liquid nitrogen and stored at -80ºC.

Germination and growth assays

To measure ABA sensitivity, seeds (circa 200 seeds per experiment) were plated on solid medium composed of MS basal salts, 1% sucrose and increasing concentrations of ABA. In order to score seed germination, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined. ABA-resistant growth from *swi3b* +/- heterozygous seedlings (circa 20 seedlings per experiment) was scored by weighting whole plants after 12 days of the transfer of 5-day-old seedlings grown on 0.5 μM ABA onto MS plates supplemented with 10 μM ABA. Heterozygous individuals from the *swi3b-1* or *swi3b-2* progeny were identified by their hygromycin or sulfadiazine resistance, respectively.

Protein extraction, protein blot analysis and immunoprecipitation

Protein extracts for immunodetection experiments were prepared from either *N. benthamiana* leaves infiltrated with *Agrobacterium* or transgenic lines from *Arabidopsis*. Plant material (~200 mg) for Western blot analysis was directly extracted in 2X Laemmli buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol, 0.001% bromophenol blue), proteins were run on a 10% SDS-PAGE gel and analyzed by immunoblotting. Plant material **(~**1 g**)** for immunoprecipitation experiments was extracted in 3 volumes of PBS buffer supplemented with 1 mM EDTA, 0.05% Triton X-100 and protease inhibitor cocktail (Roche). Protein concentration in each lysate was adjusted to the same value and equal volumes of lysates (1 ml) were incubated with 1 μg/ml of anti-HA high affinity rat monoclonal antibody (clone 3F10, Roche) for 4 h at 4ºC. After incubation, 20 μl of protein G-agarose beads (Roche) was added to precipitate the antigen-antibody complex. The protein G-agarose beads were collected after 1 h of incubation at 4ºC by centrifugation and washed three times with extraction buffer. The antigen-antibody complex was eluted by boiling in Laemmli buffer and run on a 10% SDS-PAGE gel. Proteins immunoprecipitated with anti-HA antibodies were transferred onto Immobilon-P membranes (Millipore), probed with either anti-HA-peroxidase or anti-c-myc-peroxidase conjugates (Roche) and detection was performed using the ECL advance western blotting detection kit (GE Healthcare). The imaging of the chemiluminiscent signal was achieved using a highly efficient cooled CCD camera system (LAS-3000 Luminiscent Image Analyzer from Fujifilm, Fuji Photo Film CO.,

LTD, Japan). The signal intensities of the digitalized images were quantified using Image-Gauge-V4.0 software (Fuji Photo Film CO., LTD, Japan) according to manufacturer's conditions. Immunodetection of green fluorescent protein (GFP) fusion proteins was performed with an anti-GFP monoclonal antibody (clone JL-8, Clontech) as primary antibody and ECL anti-mouse-peroxidase (GE Healthcare) as secondary antibody. A rabbit antibody against peptide comprising amino acids 3-17 of GFP (anti-GFP^N) was employed to detect YFP^N fusion proteins (Sigma G1544).

RNA analyses

Plants were grown on MS plates supplemented with 1% sucrose either in the absence or presence of 0.3 μM ABA. After 7 days, approximately 30-40 seedlings were collected and frozen in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy Plant Mini Kit and 1 μg of the RNA solution obtained was reverse transcribed using 0.1 μg oligo$(dT)_{15}$ primer and M-MLV reverse transcriptase (Roche), to finally obtain a 40 μl cDNA solution. RT-qPCR amplifications and measurements were performed using an ABI PRISM 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems). RT-qPCR amplifications were monitored using the Eva-Green™ fluorescent stain (Biotium). Relative quantification of gene expression data was carried out using the $2^{-\Delta\Delta C_T}$ or comparative C_T method (Livak and Schmittgen, 2001). Expression levels were normalized using the C_T values obtained for the *β-actin-8* gene. The presence of a single PCR product was further verified by dissociation analysis in all amplifications. All quantifications were made in triplicate on RNA samples obtained from three independent experiments. The sequences of the primers used for PCR amplifications are indicated at supplemental table.

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Supplemental Table 1. Primers used

F1261: 5´-TGACATTGTGTGCGAGGTGCT
R1560: 5´-AGTCCTTGATCTGATC AAACA
FDNPP2CA. 5´-AATTCTGTTACGGAAGCAGAGA
RPP2CA: 5´-GTCGACTTAAGACGA CGCTTGATTATTC
FATG: 5´-ATGGCCATGAAAGCTCCCGAT
R660: 5´-CGCGAATTCCTAAATCTCAACTCGTTTAAACTC
F661: 5´-CGCGAATTCTCAGTGAGGAGTCAAAGCCAGAG
R1410: 5´-CGCGAATTCCTAACACTCTATTCTATCTTC
R420: 5'-CGCGAATTC CTAGGCGGAGCTATTATAGTTGAT
F400: 5´-CGCGAATTCTCATCAACTATA ATAGCTCCGCC
FBamHI: 5´-CGCGGATCCATGGAGGAGATGACTCCC
Rno-stop: 5´-GGTTCTGGTCTTGAACTTTCT
R1407no-stop: 5'- ACACTCTATTCTATCTTCAGT
FSphI: 5´-AGCATGCAGGAG ATGACTCCCGCAGTTGCA
RSphISacI: 5´-GAGCTCGCATGCCGGTTCTGGTCTTGAACTTTCT
FPCR1: 5´-TATGATGGTCATGACGGCCATAAGGTT
RATT380: 5´-TCCGGTTCTGGGATCACAT
FATTATG: 5´-AAGCTTGTCTAATACAGCTATG
RPCR2: 5´-AACCTTATGGCCGTCATGACCATCATA

Primers used for RT-qPCR

FqRD29B: 5´ CTTGGCACCACCGTTGGGACTA
RqRD29B: 5´ TCAGTTCCCA GAATCTTGAACT
FqRAB18: 5´ TGGCTTGGGAGGAATGCTTCA
RqRAB18: 5´ CCATCGCTTGAGCTTGACCAGA
FqKIN1: 5´ GCTGGCAAAGCTGAGGAGAA
RqKIN1: 5´ TTCCCGCCTGTTGTGCTC
FqRD22: 5´ CTGTTTCCAC TGAGGTGGCTAAG
RqRD22: 5´ TGGCAGTAGAACACCGCGAAT
FqRD29A: 5´ GGAAGTGAAAGGAGGAGGAGGAA
RqRD29A: 5´ CACCACCAAACCAGCCAGATG
FqP5CS1: 5´ TGGACTTGGTGCAGAGGTGG
RqP5CS1: 5´ GAATGTCCTGATGGGT GTAAAC
FqSWI3B: 5´ CATGAGGTAGAAGGAGCAATC
RqSWI3B: 5' ACACTCTATTCTATCTTCAGT
Fqβ-actin8: 5´ AGTGGTCGTACAACCGGTATTGT
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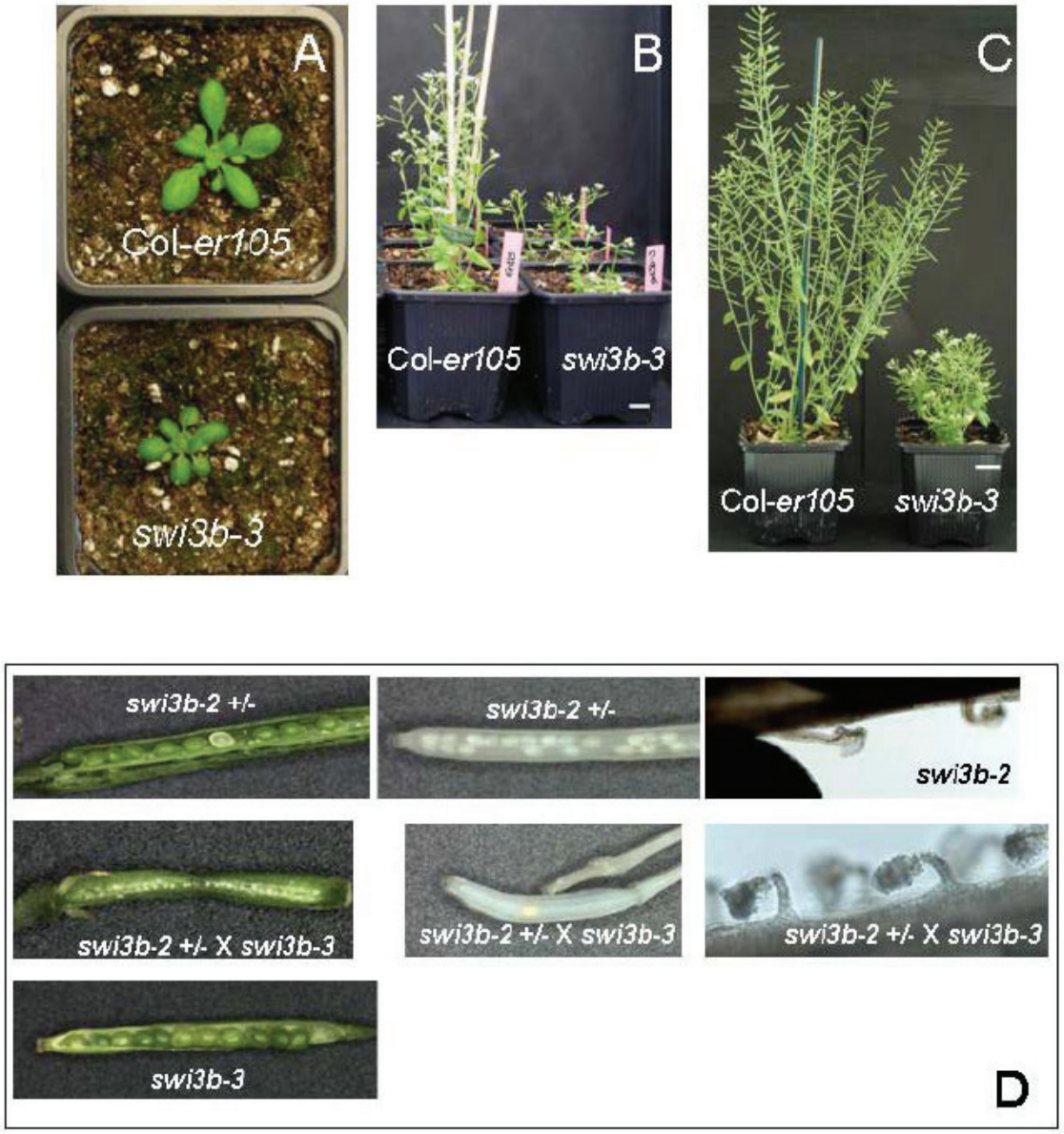
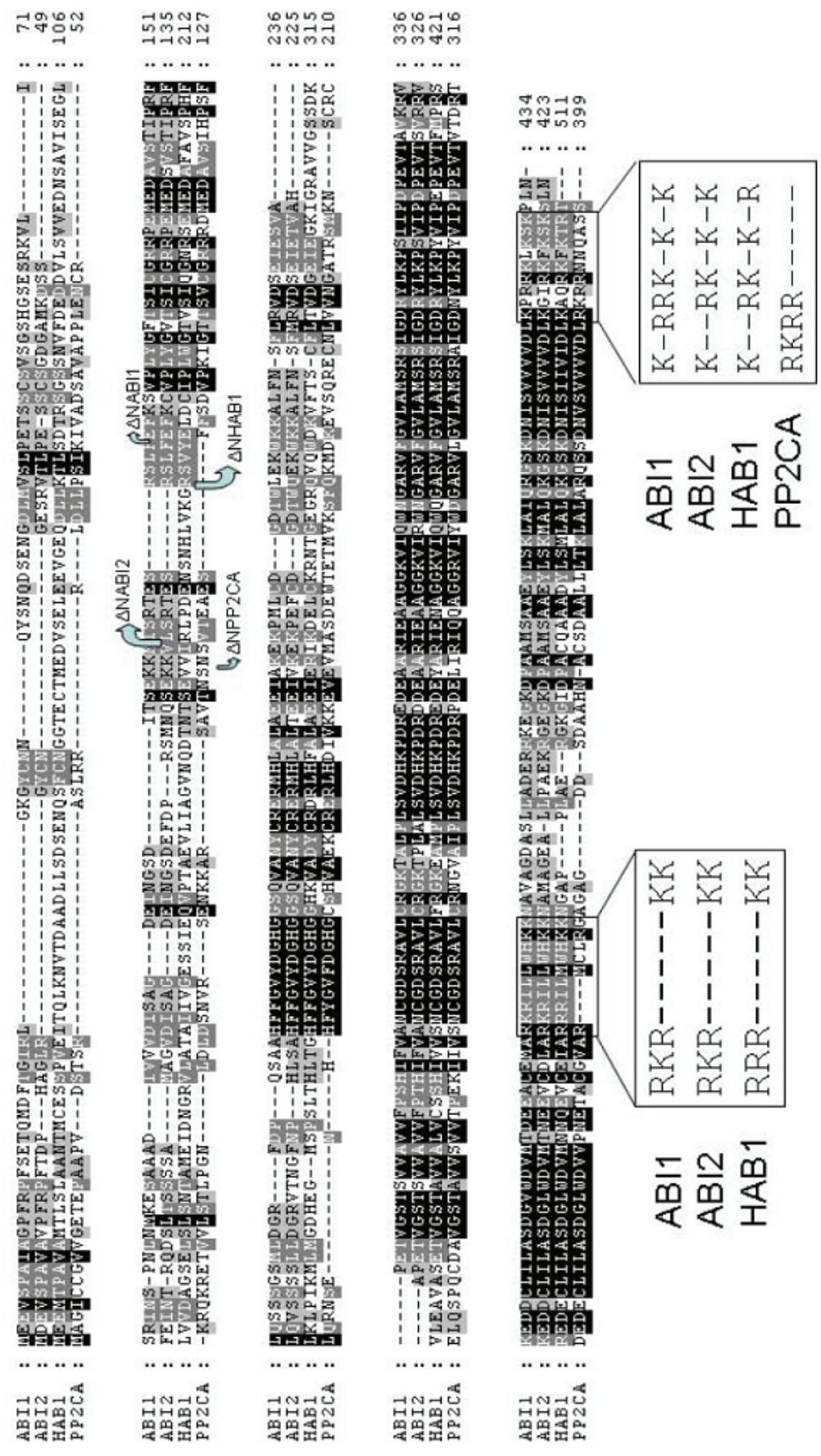


Supplemental Figure 1. Reduced vegetative and reproductive growth of the *swi3b-3* mutant. The cross between *swi3b-2* +/- and *swi3b-3* is not viable. **(A)** A representative 2-week-old plant from Col-*er105* and *swi3b-3* mutant. **(B, C)** Height, number of inflorescences and seed production were reduced in *swi3b-3* compared to Col-*er105*. Representative 6-week-old (B) and 8-week-old (C) plants from each genotype. Scale bars corresponde to 1 cm (B) and 2 cm (C). **(D)** Embryo lethality is generated by the homozygous *swi3b-2* mutation (Sarnowski et al., 2005) as well as the combination of *swi3b-2* +/- and *swi3b-3* alleles. Left panel, open green siliques. Center and right panels, detail of the embryo abortions in bleached siliques under bright field microscope..

Supplemental Figure 2. Amino acid sequence alignment of CHR12 and CHR23



Supplemental Figure 3. Putative nuclear localization signals in HAB1, PP2CA and ABI2. Localization of the N-terminal deletions generated in the different PP2Cs to avoid self-activation in the Yeast two-hybrid screen.

4. General discussion and conclusions

4. General discussion and conclusions

Abscisic acid is a phytohormone that plays a key role in the control of plant growth and development and also on plant response to stress. In this work we identified a new root specific ABA hypersensitive mutant and established a connection between HAB1, a negative regulator ABA response, and chromatin remodeling complexes.

A screen for ABA hypersensitive mutants in T-DNA lines allowed the identification of a new loss of function allele of BRX, named *brx-2*, with enhanced response to ABA-mediated inhibition of root growth. Interestingly, *brx-2* had an ABA response similar to WT in seed germination and water loss assays. This results suggest that BRX is involved in a root specific ABA response process or alternatively, that there are some functional redundancy between BRX and BRX-like genes. In the absence of exogenous ABA *brx-2* presented a short root phenotype similar to the one described for *brx-1*, that could be partially rescue by exogenous BL.

BRX has been previously implicated in a root specific control of the expression of *CPD*, a gene encoding a key enzyme of BR biosynthetic pathway, and in connecting the auxin and brassinosteroid pathways (Mouchel et al., 2006). Therefore, our results indicate a possible crosstalk between ABA and BR. This hypothesis is reinforced by the impaired ABA response of other BR mutants. As could be expected, BR biosynthetic mutants, *cpd* and *det2*, were also hypersensitive to ABA-mediated inhibition of root growth. Surprisingly, *bzr1-D*, *bes1-D* and *bsu1-D*, that show constitutive BR response, were also hypersensitive to ABA mediated root growth inhibition. Taken together these results suggest that homeostatic control of BR biosynthesis/signaling is essential for a normal response to ABA. Nevertheless, since some reports suggest that *bzr1-D* and, presumably, *bes1-D* present lower BR levels (Wang et al., 2002; Vert et al., 2005) our results can not exclude the possibility that the ABA hypersensitive phenotype observed in those BR mutants is due to a lower BR level.

The hypothesis that a branch of the BR signaling pathway directly impinges on ABA response is further supported by our striking observation that introduction of ABA-insensitivity into the *brx-2* background significantly enhances the rescue of the short root phenotype by exogenous BL treatment. This observation also raises the

hypothesis that the short root phenotype of *brx-2* could be partially related to its ABA hypersensitivity.

The existence of a possible connection between a short root phenotype and ABA hypersensitivity is also is in agreement with the phenotypical analysis of the triple, *hab1-1 abi1-2 abi2-2* and *hab1-1 abi1-2 pp2ca-1* mutants. Both triple mutants were extremely sensitive to ABA-mediated root growth inhibition and, interestingly, root growth was severely reduced in control conditions as compared to wild type.

Taken together, our physiological and phenotypic analyses reveal that disturbed regulation of root ABA sensitivity leads to inhibition of root growth.

The comparison of transcriptome response to ABA of *brx-2* and wild type, reveals that approximately 30% of ABA-upregulated and ABA-downregulated genes were differentially expressed in wild type and brx-2, suggesting that BRX has an important role in transcriptional regulation in response to ABA. These results taken together with the transcriptomic analysis of *brx-1* mutant (Mouchel et al., 2006) also indicate that ABA-treatment has a negative effect on auxin signaling in roots and, since *brx-2* itself displays impaired auxin signaling, it could be more sensitive to the ABA inhibitory effect on root growth.

Our analyses of BR biosynthesis/signaling and triple PP2Cs mutants suggest that enhanced ABA and thus stress perception might significantly contribute to the root phenotypes of various hormone pathway mutants, indicating that fine tuning of ABA response might be required to prevent a deleterious effect on growth and development in the absence of environmental stress.

Clade A PP2Cs, namely ABI1, ABI2, HAB1, HAB2, PP2CA/AHG3 and AHG1, are one of the most studied components of the ABA signaling pathway, where they act as negative regulators (e.g. Finkelstein et al, 2002; Schweighofer et al., 2004; Hirayama Shinozaki, 2007). However, the knowledge of their targets is probably far from complete.

Our results revealed a strong interaction between the catalytic domain of HAB1 and SWI3B and a weaker interaction between the catalytic domain of ABI1, ABI2 and PP2CA with SWIB3. A functional PP2C catalytic domain and both the SWIRM and ZZ zinc finger SWI3B domains appeared to be required for the interaction. The SANT

domain was dispensable for the interaction with HAB1, however, the mutations found in the *swi3b-3* and *swi3b-4* alleles provide evidence for the importance of the SANT domain for SWI3B function.

swi3b mutants show reduced sensitivity to ABA in germination, ABA-mediated root growth inhibition and reduced expression of ABA induced genes, suggesting that SWI3B is a positive regulator of ABA signaling. *hab1-1 swi3b-3* double mutant present an ABA-insensitive phenotype, similar to the *swi3b-3* one, indicating that SWI3B is epistatic to HAB1 and, therefore, HAB1 functions upstream of SWI3B in ABA signaling pathway.

HAB1 is localized both in nucleus and cytosol. However, our results clearly show that SWI3B is a nuclear target of HAB1. In the absence of ABA, ChIP experiments revealed the presence of HAB1 in the vicinity of RAB18 and RD29B promoters, suggesting a direct regulatory effect of HAB1 in the expression of ABA-mediated transcriptional regulation. Since after ABA treatment the presence of HAB1 in the vicinity of promoters of ABA-induced genes is abolished it seems that ABA-mediated removal of HAB1 from these regions is necessary for full induction of those genes.

Considering the opposed roles of HAB1 and SWI3B in ABA signaling, it is reasonable to suggest that HAB1 negatively regulates SWI3B function, modulating its role as a positive regulator of ABA signaling.

The impaired vegetative and reproductive growth phenotype of *swi3b* mutants probably reflects the important role of SWI3B in plant growth and development as a core component of SWI/SNF complexes. Other putative elements of these complexes are SWI3A, SWI3C, SWI3D, BRM, SYD, CHR12, CHR23 and BSH (Jerzmanowski, 2007). Surprisingly, *brm* and *syd* mutants presented ABA-hypersensitivity in germination, ABA-mediated root growth inhibition and enhanced expression of ABA induced genes. Probably due to functional redundancy, *chr12* and *chr23* mutants presented an ABA response similar to wild type. These results suggest that BRM and SYD are negative regulators of ABA signaling. Considering the opposite roles of SWI3B and BRM or SYD and also that these proteins are putative elements of SWi/SNF complexes we could envisage that SWI3B negatively regulates BRM and SYD.

Taking together all the results reported, a new model of HAB1, SWI3B and BRM or SYD, interaction might be suggested. We have evidence that ABA inhibits HAB1 function via ABA soluble receptors localized both at nucleus and cytosol (Rodrigues PL, 2009). We suggest the following signal transduction events: 1- ABA receptors inhibit HAB1 in an ABA-dependent manner; 2- Inhibition of HAB1 releases SWI3B function; 3- SWI3B inhibits BRM/SYD function; 4- Negative regulation of BRM/SYD is released leading to expression of ABA responsive genes (figure 1). Indeed, mutations affecting BRM cause constitutive expression of a subset of ABA responsive genes (Tang et al., 2008).

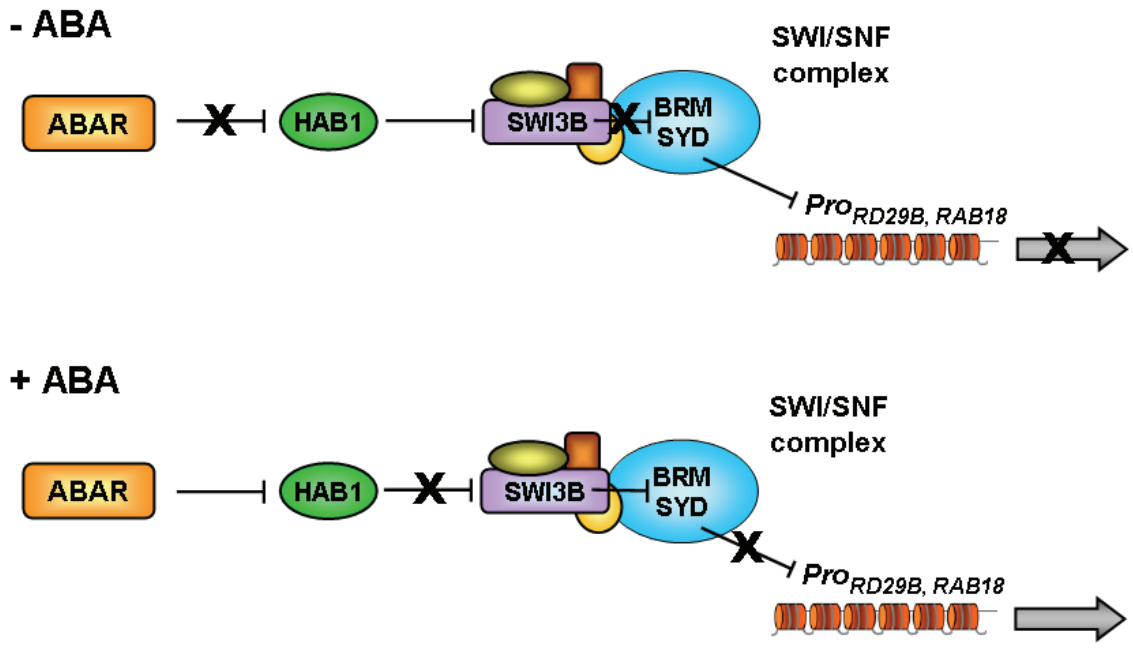


Figure 1. A model for the involvement of HAB1 and the putative members of plant SWI/SNF complex SWI3B and BRM or SYD in the regulation of plant transcriptional ABA response on the chromatin template.

5. Literature cited

(Introduction and general discussion and conclusions)

Literature cited

(Introduction and general discussion and conclusions)

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Appendix

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HAB1–SWI3B Interaction Reveals a Link between Abscisic Acid Signaling and Putative SWI/SNF Chromatin-Remodeling Complexes in *Arabidopsis* [C][W]

Angela Saez,[1] Americo Rodrigues,[1] Julia Santiago, Silvia Rubio, and Pedro L. Rodriguez[2]

Instituto de Biologia Molecular y Celular de Plantas, Consejo Superior de Investigaciones Cientificas–Universidad Politecnica de Valencia, ES-46022 Valencia, Spain

Abscisic acid (ABA) has an important role for plant growth, development, and stress adaptation. HYPERSENSITIVE TO ABA1 (HAB1) is a protein phosphatase type 2C that plays a key role as a negative regulator of ABA signaling; however, the molecular details of HAB1 action in this process are not known. A two-hybrid screen revealed that SWI3B, an *Arabidopsis thaliana* homolog of the yeast SWI3 subunit of SWI/SNF chromatin-remodeling complexes, is a prevalent interacting partner of HAB1. The interaction mapped to the N-terminal half of SWI3B and required an intact protein phosphatase catalytic domain. Bimolecular fluorescence complementation and coimmunoprecipitation assays confirmed the interaction of HAB1 and SWI3B in the nucleus of plant cells. *swi3b* mutants showed a reduced sensitivity to ABA-mediated inhibition of seed germination and growth and reduced expression of the ABA-responsive genes *RAB18* and *RD29B*. Chromatin immunoprecipitation experiments showed that the presence of HAB1 in the vicinity of *RD29B* and *RAB18* promoters was abolished by ABA, which suggests a direct involvement of HAB1 in the regulation of ABA-induced transcription. Additionally, our results uncover SWI3B as a novel positive regulator of ABA signaling and suggest that HAB1 modulates ABA response through the regulation of a putative SWI/SNF chromatin-remodeling complex.

INTRODUCTION

The phytohormone abscisic acid (ABA) is a key regulator of plant growth and development as well as plant responses to decreased water availability. A fast mechanism to adjust ABA levels and respond to changing environmental cues is the hydrolysis of glucose-conjugated ABA (Lee et al., 2006). Additionally, water stress leads to the accumulation of ABA through enhanced expression of ABA biosynthetic genes, mainly *9-cis-epoxycarotenoid dioxygenase3* (Nambara and Marion-Poll, 2005; Barrero et al., 2006). ABA triggers a variety of adaptive responses, such as stomatal closure and differential gene expression, which are crucial for plant survival under stress conditions (Schroeder et al., 2001; Nambara and Marion-Poll, 2005).

Decades of research in ABA signaling have resulted in the identification of many elements of the ABA signal transduction pathway, including both negative and positive regulators (reviewed in Finkelstein et al., 2002; Himmelbach et al., 2003; Israelsson et al., 2006). Under water stress, ABA signaling leads to coordinated remodeling of gene expression, which affects more than ~5% of the plant transcriptome (Huang et al., 2007). Downstream nuclear effects of ABA are mediated by different transcription factors (TFs) that play a positive role in ABA signaling, which comprise ABA-responsive element binding proteins (ABI5/ABF/AREB/bZIP family) (Choi et al., 2000; Finkelstein and Lynch, 2000; Uno et al., 2000; Bensmihen et al., 2002), *Arabidopsis thaliana* ABI3 and maize (*Zea mays*) VP1 TFs of the B3 domain family (McCarty et al., 1991; Giraudat et al., 1992), the ABI4 TF from the APETALA2 domain family (Finkelstein et al., 1998), and ATMYC2 and ATMYB2 TFs (Abe et al., 2003). Some TFs that function as transcriptional repressors of ABA response have also been described (Himmelbach et al., 2002; Pandey et al., 2005; Song et al., 2005). In eukaryotes, the packaging of DNA into chromatin implies that both transcriptional activators and repressors work together with large multisubunit complexes that remodel nucleosomes to regulate gene expression (Carrozza et al., 2003; Smith and Peterson, 2005). Two general classes of chromatin-modifying factors can be distinguished, those that covalently modify the N-terminal tails of histone proteins and those that utilize ATP hydrolysis to remodel or reposition nucleosomes (Carrozza et al., 2003; Smith and Peterson, 2005). The first class includes protein complexes that acetylate or deacetylate Lys residues present in the N termini of histone proteins (histone acetyltransferases) and histone deacetylases. The second class of factors is composed of ATP-dependent chromatin-remodeling complexes, which alter nucleosome structure or positioning. Among them, the yeast SWI/SNF complex was the first one to be described (Cairns et al., 1994; Peterson et al., 1994). In addition to the ATPase Swi2/Snf2, it contains a central core composed of three additional polypeptides, Swi3, Snf5, and

[1] These authors contributed equally to this work.

[2] Address correspondence to prodriguez@ibmcp.upv.es.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Pedro L. Rodriguez (prodriguez@ibmcp.upv.es).

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Swp73, which are required for the assembly and activity of the complex (Cairns and Kingston, 2000; Smith and Peterson, 2005; Yang et al., 2007). Some reports of chromatin-modifying factors that affect ABA responses have been published (Song et al., 2005; Sridha and Wu, 2006); however, taking into account the deep impact of ABA on the regulation of gene expression and the many TFs involved in this process, we can envisage that many elements in this field are yet to be discovered.

Protein phosphatase type 2Cs (PP2Cs) were identified as key components of ABA signaling from pioneering work with the ABA-insensitive *abi1-1* and *abi2-1* mutants (Koornneef et al., 1984; Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997; Rodriguez et al., 1998a). Currently, at least six *Arabidopsis* PP2Cs, namely ABI1, ABI2, PP2CA/AHG3, ABA-HYPERSENSITIVE GERMINATION1 (AHG1), HYPERSENSITIVE TO ABA1 (HAB1), and HAB2, are known to regulate ABA signaling. Genetic approaches indicate that these PP2Cs are negative regulators of ABA signaling (Gosti et al., 1999; Merlot et al., 2001; Tahtiharju and Palva, 2001; Gonzalez-Garcia et al., 2003; Leonhardt et al., 2004; Saez et al., 2004, 2006; Kuhn et al., 2006; Yoshida et al., 2006b; Nishimura et al., 2007). Although interacting partners for some of these PP2Cs have been described (Cherel et al., 2002; Guo et al., 2002; Himmelbach et al., 2002; Ohta et al., 2003; Miao et al., 2006; Yang et al., 2006; Yoshida et al., 2006a), the overall knowledge of their targets and their role in ABA signaling is far from complete. In this work, we have pursued a two-hybrid approach using the PP2C HAB1 as bait to identify putative interacting preys. Interestingly, a prevalent interacting partner of HAB1 was found to be the SWI3B protein, which is an *Arabidopsis* homolog of the SWI3 core subunit of SWI/SNF chromatin-remodeling complexes (Sarnowski et al., 2002; Zhou et al., 2003). These complexes, already characterized in yeast, *Drosophila*, and mammals, have not yet been biochemically characterized in plants, although genome analysis suggests that *Arabidopsis* contains the active components required to form such complexes (Farrona et al., 2004; Sarnowski et al., 2005). Thus, in *Arabidopsis*, four SWI3-like proteins (i.e., SWI3A, SWI3B, SWI3C, and SWI3D) have been identified (Sarnowski et al., 2002; Zhou et al., 2003) as well as other putative components of SWI/SNF complexes (Brzeski et al., 1999; Farrona et al., 2004; Bezhani et al., 2007). Current data on loci that encode putative components of SWI/SNF chromatin-remodeling complexes show that they operate as modifiers of transcriptional or epigenetic regulation in plant growth and development (Kwon and Wagner, 2007). Our data provide a link between a component of the ABA signaling pathway and a putative component of SWI/SNF chromatin-remodeling complexes and, therefore, suggest that these complexes are also involved in the hormonal response to abiotic stress.

RESULTS

Identification of SWI3B as a HAB1-Interacting Partner

A yeast two-hybrid screen was used to identify proteins that interact with the PP2C HAB1. Preliminary experiments revealed that full-length HAB1 fused to the GAL4 DNA binding domain (GBD) resulted in the activation of *HIS3* and *ADE2* reporters from the AH109 yeast strain used in this study (see Supplemental Figure 1 online). N-terminal truncation of some clade A PP2Cs (Schweighofer et al., 2004) is required to reduce their potential to activate transcription (Himmelbach et al., 2002; this work). Indeed, the N-terminal 1 to 180 amino acid residues either from HAB1 (see Supplemental Figure 1 online) or from the closely related PP2C HAB2, when fused to the GBD, generated a powerful transcriptional activator. Thus, only the catalytic region (amino acid residues 179 to 511) of the PP2C HAB1 (ΔNHAB1) was used as a bait to screen an *Arabidopsis* expression library containing random cDNAs fused to the GAL4 activation domain (GAL) in the pACT2 vector (Nemeth et al., 1998). This N-terminal truncation of HAB1 showed approximately twofold higher phosphatase activity than full-length HAB1 (Figure 1B). From 10^6 colonies screened, 20 positive clones that showed autotrophic growth in medium lacking both adenine and His were selected. Sequence analysis of the recovered pACT2 clones revealed that 11 of the 20 putative interacting preys contained the full-length cDNA from *SWI3B*. Therefore, these results indicate that AtSWI3B is a prevalent HAB1-interacting partner in a two-hybrid screening.

The Interaction of HAB1 and SWI3B Requires a Functional PP2C Catalytic Domain and Maps to the N-Terminal Half of SWI3B

Protein domain analysis using the PFAM database of global domain hidden Markov models and different pattern and profile searches in Expasy (http://www.expasy.org) served to identify SWIRM (48 to 136), SANT (224 to 272), and Leu zipper domains (399 to 452) in the SWI3B amino acid sequence, in agreement with previous findings from Sarnowski et al. (2005). Additionally, we could identify a ZZ zinc finger domain (Cys-x_2-Cys motifs plus a conserved YDL motif) between amino acid residues 169 and 208. A similar ZZ zinc finger domain was identified in *Arabidopsis* SWI3C by Hurtado et al. (2006). In order to determine specific regions of SWI3B involved in the interaction with ΔNHAB1, different deletions of the *SWI3B* coding sequence in the prey vector pACT2 were generated. Previously, we confirmed that a combination of the empty pGBT9 plasmid and pACT2-*SWI3B* did not activate transcription of the *HIS3* and *ADE2* reporter genes (Figure 1A); moreover, none of the deletion constructs activated transcription in the absence of bait protein interactors. In combination with the bait construct pGBT9-*ΔNHAB1*, the deletion constructs C1 and C2 activated transcription of the reporter genes to the same levels as full-length *SWI3B* (Figure 1A). This result mapped the HAB1-interacting domain to the first 220 amino acid residues of SWI3B. In agreement with this result, the prey construct N1 did not activate the reporter genes in the growth assay. Further attempts to delimit the minimal region of SWI3B that interacted with ΔNHAB1 failed, as additional deletions affecting the N-terminal half of SWI3B (SWIRM and ZZ prey constructs) eliminated the interaction with ΔNHAB1.

In order to clarify the specificity of the interaction, we examined whether other SWI3-like proteins from *Arabidopsis* showed interaction with ΔNHAB1. In contrast with SWI3B, none of the SWI3A, SWI3C, or SWI3D proteins interacted with ΔNHAB1

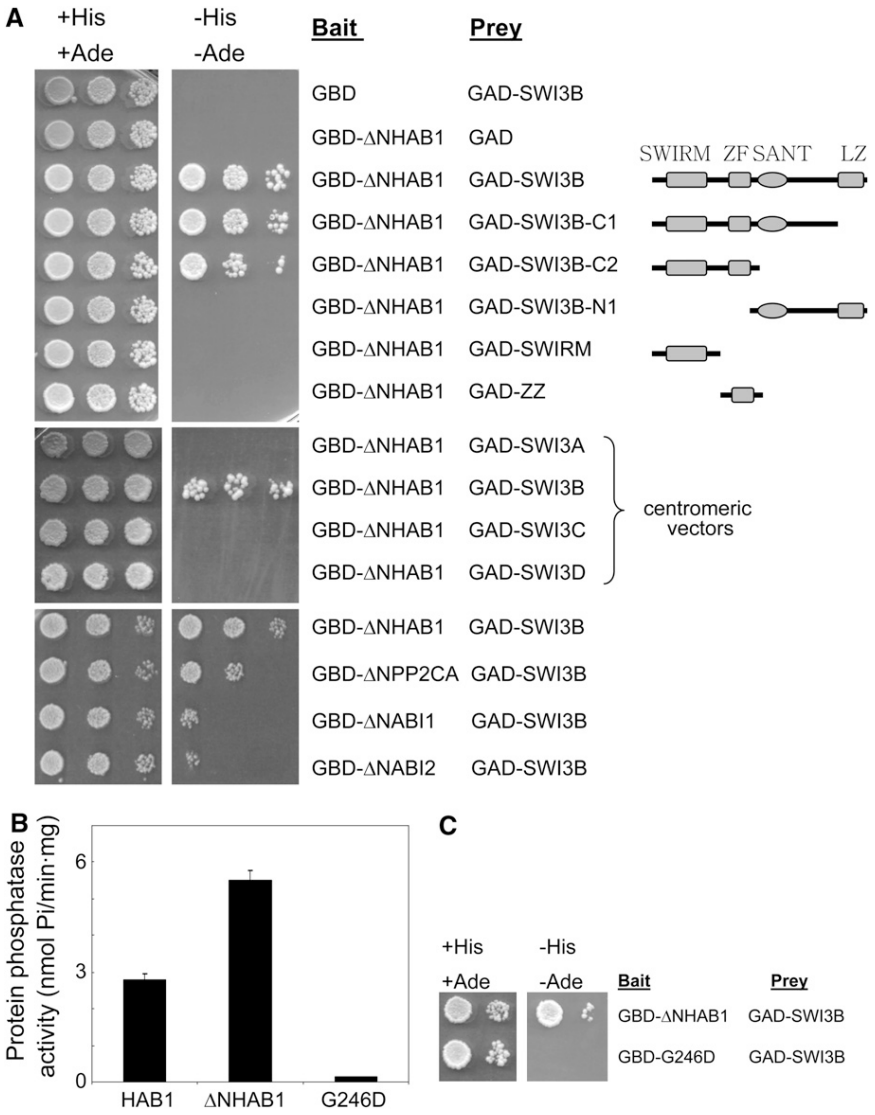

Figure 1. HAB1 and SWI3B Interact in a Yeast Two-Hybrid Assay.

Interaction was determined by growth assay on medium lacking His and adenine. Dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of saturated cultures were spotted onto the plates.

(A) Top, interaction assay with ΔNHAB1 as bait (fused to the GBD) and either full-length or different deletions of SWI3B as putative preys (fused to the GAD). Schemes of SWI3B domains and the different protein deletions are shown. Deletions C1 and C2 lacked C-terminal amino acid residues 346 to 469 and 221 to 469, respectively. Deletion N1 lacked N-terminal amino acid residues 1 to 220. GAD-SWIRM and GAD-ZZ comprised amino acid residues 1 to 140 and 134 to 220, respectively. Middle, interaction assay with SWI3A, SWI3B, SWI3C, and SWI3D as putative preys. Bottom, interaction assay with ΔNPP2CA, ΔNABI1, and ΔNABI2 as baits and SWI3B as prey.

(B) Protein phosphatase activity of MBP-HAB1, MBP-ΔNHAB1, and MBP-G246D ΔNhab1 fusion proteins. Values are averages ± SE from three independent experiments.

(C) Interaction assay with ΔNHAB1 and G246D ΔNhab1 as baits and SWI3B as prey.

(Figure 1A, middle). This result highlights the remarkable functional diversification previously described for the four SWI3-like proteins from *Arabidopsis* (Sarnowski et al., 2005; Hurtado et al., 2006). HAB1 belongs to a group of PP2Cs (clade A; Schweighofer et al., 2004) in which six of the identified genes are associated with ABA signaling. Gene expression data and genetic analysis indicate that HAB1, PP2CA, ABI1, and ABI2 play a predominant role in ABA signaling in both seeds and vegetative tissue (Saez et al., 2004, 2006; Kuhn et al., 2006; http://www.genevestigator.ethz.ch). Therefore, we generated N-terminal truncations of PP2CA, ABI1, and ABI2 fused to GBD and their interaction with SWI3B was examined (Figure 1A, bottom). ΔNPP2CA, ΔNABI1,

and ΔNABI2 were able to interact with SWI3B, although it was apparent in the growth assay that the interaction was weaker than that observed for ΔNHAB1. All fusion proteins were expressed at similar levels, as verified by protein gel blot analysis using antibodies against the GAD and GBD. Finally, in order to examine the role of the catalytic PP2C domain in the interaction with SWI3B, a point-mutated version of HAB1 that replaced Gly-246 for Asp (G246D hab1) was introduced in the two-hybrid test. The Gly-246 is localized in a conserved motif from eukaryotic PP2Cs, and its replacement by Asp interferes with Mg^{2+} binding and strongly impairs PP2C activity (Leung et al., 1994; Meyer et al., 1994). Indeed, both G246D hab1 (Robert et al., 2006) and G246D ΔNhab1 (Figure 1B) show <3% in vitro activity than the wild type. Interestingly, G246D ΔNhab1 did not interact with SWI3B in the two-hybrid assay, indicating that a functional catalytic domain of HAB1 is required for its interaction with SWI3B (Figure 1C).

Subcellular Localization of HAB1 and SWI3B

To determine the subcellular localization of HAB1 and SWI3B proteins in plant cells, we performed in vivo targeting experiments in tobacco (*Nicotiana benthamiana*). To this end, *35S:HAB1-GFP* and *35S:SWI3B-GFP* constructs were generated and delivered into leaf cells of tobacco by *Agrobacterium tumefaciens* infiltration (Voinnet et al., 2003). Coexpression of bZIP63-YFP^N and bZIP63-YFP^C served as a positive control for localization of a nuclear protein (Walter et al., 2004). In the case of SWI3B, strong green fluorescent protein (GFP) fluorescence was observed in the nucleus of tobacco cells, whereas HAB1 localized in both the nucleus and the cytosol (Figure 2A). Similar results were obtained when the coding region of *HAB1* was fused to the C-terminal end of *GFP* (see Supplemental Figure 2 online). Finally, ABA treatment did not modify the subcellular localization of either HAB1-GFP or SWI3B-GFP under our experimental conditions (Figure 2A; see Supplemental Figure 2 online).

In addition to using GFP fusions, we examined the subcellular localization of HAB1 by standard biochemical techniques. To this end, we generated transgenic lines (in a *hab1-1* background) that expressed a double hemagglutinin (HA) epitope–tagged version of HAB1 (HAB1-dHA) under the control of the *HAB1* native promoter. HAB1-dHA efficiently complemented the ABA-hypersensitive phenotype of *hab1-1* in germination assays (Saez et al., 2004). HAB1-dHA could be detected in both the cytosolic and nuclear fractions (Figure 2B). Washing of the nuclei/organelles with a buffer containing 0.5% Triton X-100 released a significant amount of HAB1-dHA (W fraction), which we assume to be of nuclear origin according to the localization of HAB1-GFP and GFP-HAB1 fusions. Additionally, a fraction of HAB1-dHA was associated with the nuclear insoluble fraction, which contains the major histones and is mostly composed of chromatin (Poveda et al., 2004; Cho et al., 2006). Finally, HAB1-dHA was also detected in a nuclear soluble fraction that was obtained by rupture of the nuclei and extraction with a buffer containing 0.4 M NaCl. An estimation of the cytosolic:nuclear HAB1 ratio was made based on protein blot analysis with the anti-HA antibody. Figure 2C shows an approximately threefold difference in HAB1 abundance between the cytosolic and combined nuclear fractions. Taking into account the additional fourfold enrichment during the nuclei isolation process before protein gel loading, the HAB1 cytosolic:nuclear ratio was ~12:1. Treatment with 50 μM ABA for 1 h did not substantially modify this ratio (Figure 2C).

In Planta Interaction between HAB1 and SWI3B

Bimolecular fluorescence complementation (BiFC) assays were used to detect the interaction between HAB1 and SWI3B in plant cells. To this end, HAB1 was translationally fused to the C-terminal 84–amino acid portion of yellow fluorescent protein (YFP^C) in the pSPYCE vector, which generated a HAB1-epitope HA-YFP^C fusion protein (Figure 3B). For the other partner, the N-terminal half of SWI3B was translationally fused to the N-terminal 155–amino acid portion of yellow fluorescent protein (YFP^N) in the pSPYNE vector, which generated a SWI3B-epitope myc-YFP^N fusion protein (Figure 3B). The corresponding constructs were codelivered into leaf cells of tobacco by *Agrobacterium* infiltration and, as a result, fluorescence was observed in the nucleus of tobacco cells (Figure 3A, left). No fluorescence signal was observed when pSPYCE-*HAB1* vector was codelivered with pSPYNE or when pSPYNE-*SWI3B* was codelivered with pSPYCE. Moreover, in agreement with the previous finding in the two-hybrid assay, introduction of the G246D mutation in the sequence of HAB1 abolished the interaction with SWI3B in the BiFC assay (Figure 3A, right).

In addition to the observed BiFC fluorescent signal, we confirmed the interaction by coimmunoprecipitation of HAB1 and SWI3B in tobacco protein extracts prepared from the BiFC assay described above (Figure 3B). HAB1 and SWI3B can be coimmunoprecipitated, as we could detect SWI3B in the immunocomplex precipitated with an antibody to epitope HA, which pulls down the HAB1-HA-YFP^C fusion protein (Figure 3B). By contrast, introduction of the G246D mutation in the sequence of HAB1 prevented the coimmunoprecipitation of SWI3B (Figure 3B). Thus, results from two in planta assays support the interaction between HAB1 and SWI3B.

Finally, BiFC assays showed that PP2CA, ABI1, and ABI2 were able to interact with SWI3B in the nucleus of tobacco cells (Figure 3C). Expression of fusion proteins was verified by protein gel blot analysis using antibodies against the epitope HA and peptide comprising amino acids 3 to 17 of GFP (anti-GFP^N) (Figure 3D). ABA treatment (50 μM for 1 h) did not change the interaction of the PP2Cs and SWI3B. However, complex formation in BiFC is essentially irreversible, which prevents the imaging of changes in the protein association state (Fricker et al., 2006).

swi3b Mutants Show a Reduced Sensitivity to ABA and Reduced Expression of RD29B and RAB18

The *swi3b-1* and *swi3b-2* knockout mutants (Figure 4A) were previously reported to be embryo-lethal (Sarnowski et al., 2005); therefore, we decided to examine heterozygous mutants for phenotypic effects. Phenotypic effects caused by gene haploinsufficiency (monoallelic expression and heterozygosis) have been described in mutants affected in diverse components of the chromatin-remodeling machinery (Bultman et al., 2000; Roberts et al., 2000; Alarcon et al., 2004; David et al., 2006).

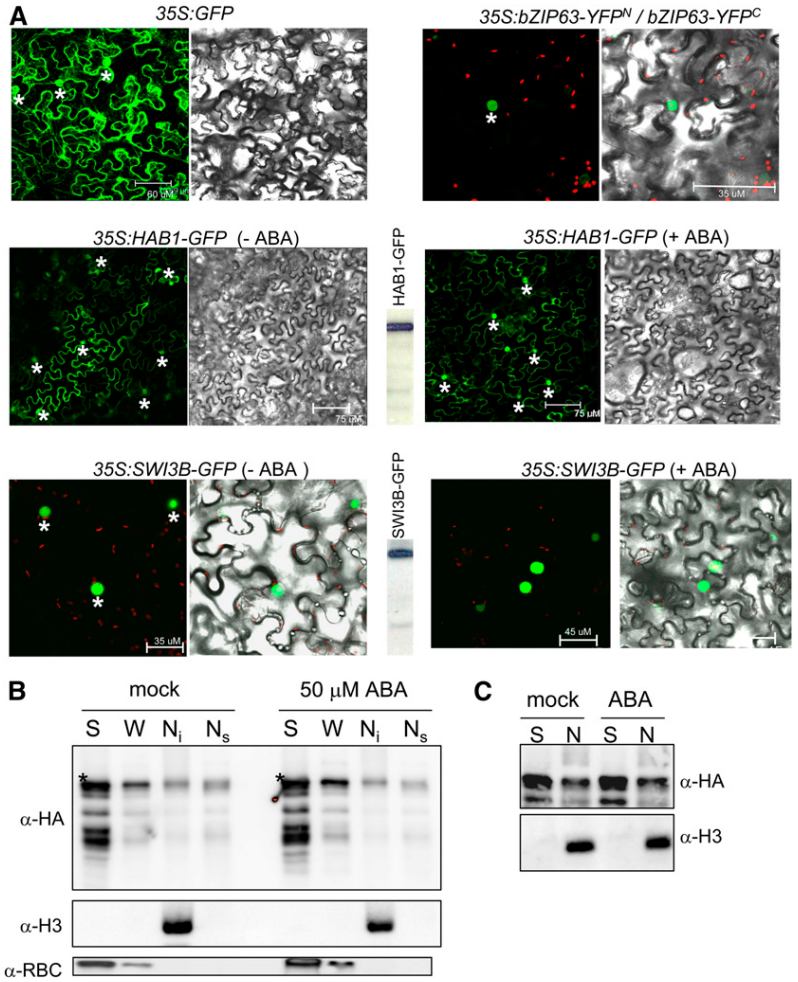

Figure 2. HAB1 Localizes at Both Cytosol and Nucleus.

(A) Subcellular localization of HAB1 and SWI3B in *Agrobacterium*-infiltrated tobacco leaves. Epifluorescence and bright-field images of epidermal leaf cells infiltrated with a mixture of *Agrobacterium* suspensions harboring the indicated constructs and the silencing suppressor p19. BiFC-induced bZIP63 dimerization served to identify the nuclei of tobacco epidermal cells (asterisks). The expression of the proteins is demonstrated by immunodetection with anti-GFP for HAB1-GFP and SWI3B-GFP (center, between panels). Treatment with 50 μM ABA for 1 h (+ABA) did not change the subcellular localization of both HAB1-GFP and SWI3B-GFP.

(B) Biochemical fractionation of HAB1-dHA (full-length protein marked with asterisks). Plant material was obtained from the *hab1-1::ProHAB1-HAB1-dHA* transgenic line after mock treatment or treatment with 50 μM ABA for 1 h. The soluble cytosolic fraction (S), nuclei/organelles wash fraction (W), nuclear insoluble fraction (Ni), and nuclear soluble fraction (Ns) were analyzed using anti-HA, anti-histone 3 (H3), and anti–ribulose-1,5-bis-phosphate carboxylase/oxygenase (RBC) antibodies.

(C) Relative amount of HAB1-dHA in the soluble cytosolic (S) and nuclear (N) fractions after mock treatment or treatment with 50 μM ABA for 1 h.

For instance, heterozygous mice that have a single copy of either *BRG1* (the mammalian orthologous gene of the yeast Swi2/Snf2 ATPase) or *SNF5* (a core component of the SWI/SNF complex) are predisposed to different tumors, indicating that a full dosage of both BRG1 and SNF5 is required for proper control of gene expression and tumor suppression (Bultman et al., 2000; Roberts et al., 2000). Therefore, we decided to analyze ABA responsiveness in the progeny of *Arabidopsis swi3b* +/− seedlings, which represents an ~2:1 mixture of heterozygous and wild-type seeds (Sarnowski et al., 2005). Thus, the progeny from *swi3b-1* and *swi3b-2* heterozygous plants were analyzed to score ABA-mediated inhibition of germination and growth. These assays revealed a reduced sensitivity to ABA of *swi3b* +/− seeds and seedlings compared with the wild type (Figures 4B and 4C). This phenotype was particularly apparent in growth assays, as after 10 d in 10 μM ABA both *swi3b-1* and *swi3b-2* +/− seedlings showed ~80 to 90% higher weight than wild-type seedlings (Figure 4C). By contrast, water-loss assays did not show significant differences between wild-type and *swi3b-1* and *swi3b-2* +/− plants (see Supplemental Figure 3A online). Nevertheless, since ~50% reduction in the expression of *SWI3B* (see Supplemental Figure 3B online) led to reduced sensitivity to ABA in germination

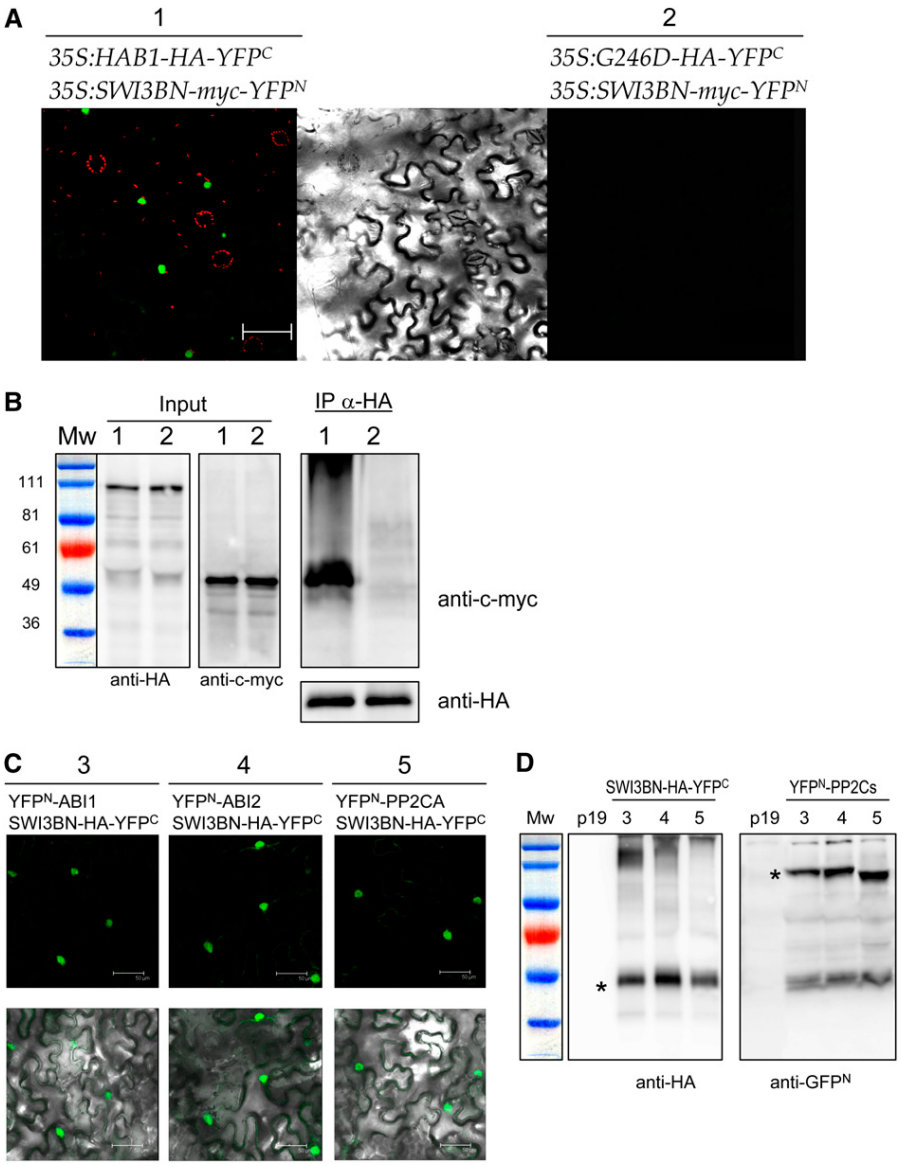

Figure 3. BiFC Visualization and Coimmunoprecipitation Experiments Show Interaction between HAB1 and SWI3B in the Nucleus of Tobacco Leaves.

(A) Introduction of the G246D substitution into HAB1 abolishes the interaction with SWI3B. Epifluorescence and bright-field images of epidermal leaf cells infiltrated with a mixture of *Agrobacterium* suspensions harboring constructs HAB1-HA-YFP^C/SWI3BN-myc-YFP^N (panel 1) or G246D-HA-YFP^C/SWI3BN-myc-YFP^N (panel 2) and the silencing suppressor p19. The bar corresponds to 75 μm. Green color corresponds to YFP, whereas red color is generated by chlorophyll fluorescence.

(B) Coimmunoprecipitation assay demonstrates the interaction between HAB1 and SWI3B in planta. Protein extracts obtained from tobacco leaves infiltrated with *Agrobacterium* suspensions harboring constructs HAB1-HA-YFP^C/SWI3BN-myc-YFP^N (lanes 1) or G246D-HA-YFP^C/SWI3BN-myc-YFP^N (lanes 2) were analyzed using anti-HA or anti-c-myc antibodies. Input levels of epitope-tagged proteins in crude protein extracts (20 μg of total protein) were analyzed by immunoblotting. Immunoprecipitated epitope HA–tagged proteins were probed with anti-c-myc antibodies to detect coimmunoprecipitation of SWI3BN-myc-YFP^N with HAB1-HA-YFP^C.

(C) BiFC assays show the interaction of ABI1, ABI2, and PP2CA with SWI3B in the nucleus of tobacco leaves. Cells were infiltrated with a mixture of *Agrobacterium* suspensions harboring constructs SWI3BN-HA-YFP^C/YFP^N-ABI1 (panel 3), SWI3BN-HA-YFP^C/YFP^N-ABI2 (panel 4), or SWI3BN-HA-YFP^C/YFP^N-PP2CA (panel 5) and the silencing suppressor p19.

(D) Protein gel blot analysis demonstrates the expression of SWI3BN-HA-YFP^C and the corresponding YFP^N-PP2Cs (asterisks). Protein extracts obtained from tobacco leaves infiltrated with *Agrobacterium* suspensions harboring the silencing suppressor p19 and constructs SWI3BN-HA-YFP^C/YFP^N-ABI1 (lane 3), SWI3BN-HA-YFP^C/YFP^N-ABI2 (lane 4), SWI3BN-HA-YFP^C/YFP^N-PP2CA (lane 5), or p19 alone (lane p19) were analyzed using anti-HA or anti-GFP^N antibodies.

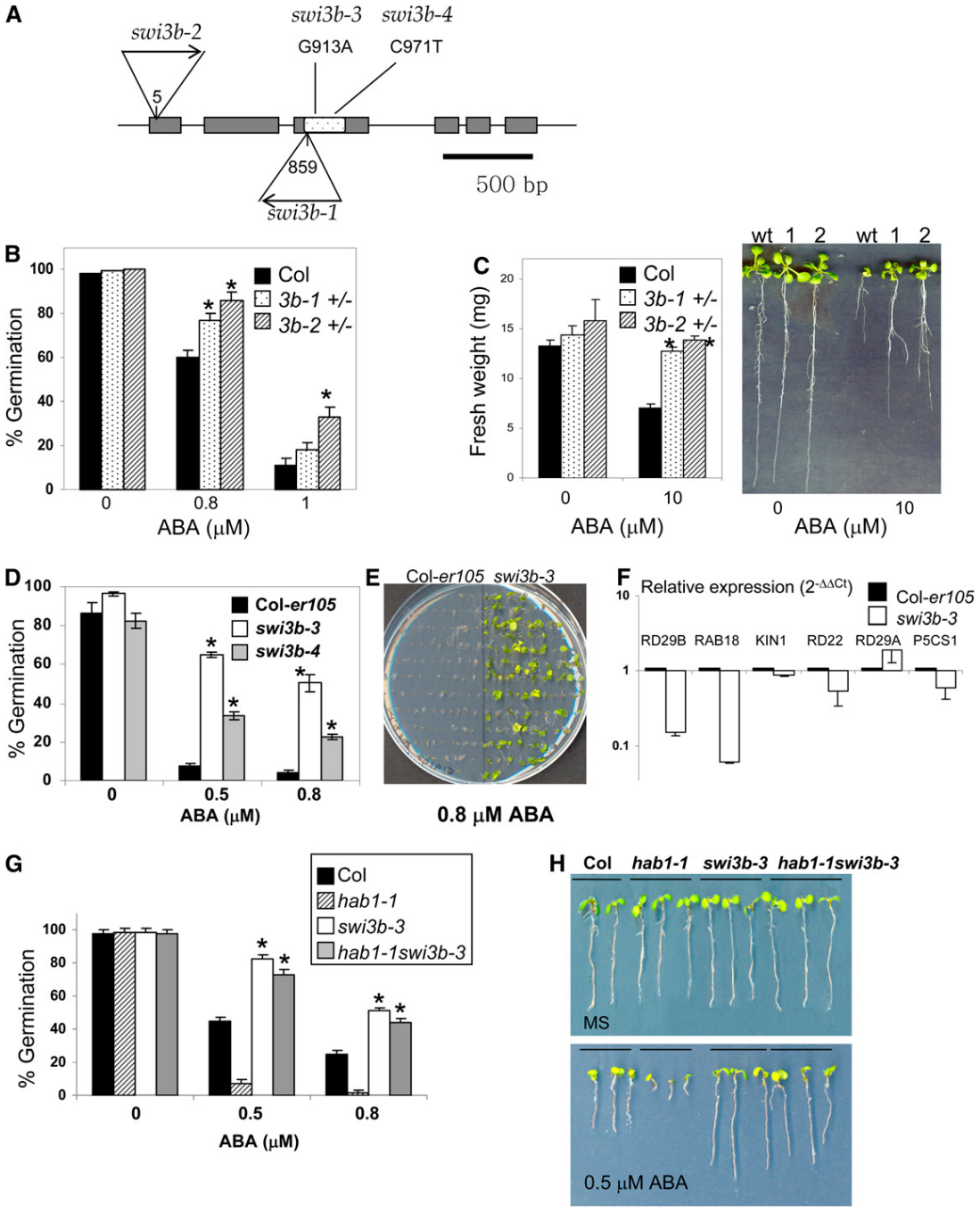

Figure 4. *swi3b* Mutants Show Reduced Sensitivity to ABA-Mediated Inhibition of Germination and Growth.

(A) T-DNA insertions in the *swi3b-1* and *swi3b-2* alleles and localization of ethyl methanesulfonate–induced mutations in *swi3b-3* and *swi3b-4* alleles. The numbering begins at the ATG translation start codon. The gray boxes represent exons. The SANT domain is spotted within the third exon.
(B) ABA effects on germination in the progeny of *swi3b-1* and *swi3b-2* heterozygous plants. The percentage of seeds that germinated and developed green cotyledons in the presence of the indicated concentrations of ABA is shown. Values are averages ± SD for three independent experiments (n = 200 seeds per experiment). * $P < 0.01$ (Student's t test) when comparing data from each genotype and the wild type in the same assay conditions.
(C) Reduced sensitivity of *swi3b-1* and *swi3b-2* heterozygous (+/−) seedlings to ABA-mediated growth inhibition. Fresh weight was measured in 12-d-old seedlings grown in MS medium either lacking or containing 10 μM ABA. Values are averages ± SD for three independent experiments (n = 20 seedlings per experiment). Representative seedlings of Col (wild type [wt]), *swi3b-1* +/− (1), and *swi3b-2* +/− (2) were removed from medium lacking or containing 10 μM ABA and rearranged on agar plates (at right).

and growth assays, these results suggest that *SWI3B* is a positive regulator of ABA signaling.

Further evidence of the role of SWI3B in ABA signaling was obtained through the analysis of point mutations in *swi3b* alleles that were recovered by the *Arabidopsis* TILLING (for targeting-induced local lesions in genomes) program (http://tilling.fhcrc.org:9366/home.html) in a Columbia (Col)–*er105* background. Thus, two new *swi3b* alleles were identified, *swi3b-3* and *swi3b-4*, which resulted in the substitution of Asp-245 by Asn and Ser-264 by Phe, respectively. Both Asp-245 → Asn and Ser-264 → Phe mutations are localized in the SANT domain of SWI3B and, according to SIFT (for sorting intolerant from tolerant) software analysis, are predicted to affect protein function (SIFT score < 0.05) (Ng and Henikoff, 2001). Analysis of ABA-mediated inhibition of germination in *swi3b-3* and *swi3b-4* revealed that both mutants showed a reduced sensitivity to ABA in this assay compared with the Col-*er105* background (where TILLING mutants were originated) (Figure 4D). In particular, the *swi3b-3* mutant also showed a reduced sensitivity to ABA-mediated inhibition of early growth (Figure 4E). These results, together with those of *swi3b-1* and *swi3b-2* +/− seedlings, show that SWI3B is a positive regulator of ABA signaling that mediates the ABA response in seeds and vegetative tissue.

Additionally, we wondered whether SWI3B might play a role in the regulation of gene expression in response to ABA. SWI3B is a putative core component of SWI/SNF complexes, and chromatin remodelers have a well-established role in transcriptional regulation. Therefore, real-time quantitative polymerase chain reaction (RT-qPCR) was used to analyze the expression of the ABA-responsive *RD29B*, *RAB18*, *KIN1*, *RD22*, *RD29A*, and *P5CS1* genes in the wild type and the *swi3b-3* mutant (Figure 4F). In general terms, these gene markers show low expression in the absence of ABA or stress treatment, which is upregulated in response to the inductive signal. Upon ABA treatment, expression of *RD29B* and *RAB18* in *swi3b-3* was 15 and 6%, respectively, of that found in the wild type, whereas expression of the other gene markers did not differ more than twofold in both genotypes. Thus, SWI3B appears to regulate a subset of ABA-inducible genes, whereas its function seems to be partially dispensable or redundant for the expression of other ABA-responsive genes. Finally, to further characterize the genetic relationship between the ABA-hypersensitive locus *hab1-1* and the ABA-insensitive locus *swi3b-3*, we generated a *hab1-1swi3b-3* double mutant. Analysis of ABA-mediated inhibition of germination (Figure 4G) and early seedling growth (Figure 4H) revealed that *hab1-1swi3b-3* showed an ABA-insensitive phenotype, in contrast with *hab1-1*, which indicates that SWI3B is epistatic to HAB1; therefore, HAB1 functions upstream of SWI3B in the ABA signaling pathway. In addition to reduced sensitivity to ABA in the assays described above, the *swi3b-3* allele showed both impaired vegetative and reproductive growth (see Supplemental Figure 4 online), which likely reflects the key role of SWI3B in plant growth and development as a core component of diverse SWI/SNF complexes (Zhou et al., 2003; Sarnowski et al., 2005; Bezhani et al., 2007). In agreement with this role, combination of the *swi3b-3* and *swi3b-2* alleles was embryo-lethal (see Supplemental Figure 4 online).

The Presence of HAB1 in the Vicinity of the ABA-Responsive RD29B and RAB18 Promoters Is Abolished by ABA

The interaction of HAB1 and SWI3B as well as the phenotype of *swi3b* mutants suggest that HAB1 modulates ABA response through the regulation of a putative SWI/SNF chromatin-remodeling complex. In order to analyze the presence of HAB1 in plant chromatin and the putative influence of ABA on it, we performed chromatin immunoprecipitation (ChIP) experiments. To this end, we used the *hab1-1* transgenic line complemented by HAB1-dHA described above and demonstrated that HAB1-dHA could be immunoprecipitated using a monoclonal antibody to HA peptide (Figure 5A). ChIP experiments were performed on formaldehyde–cross-linked chromatin extracted from either *hab1-1* or *hab1-1::ProHAB1-HAB1-dHA* plants. Genomic DNA fragments that coimmunoprecipitated with HAB1-dHA were analyzed by RT-qPCR (Figures 5B to 5D). To this end, we used different primer pairs that covered the promoters of the ABA-responsive genes *RD29B* and *RAB18* as well as a control gene, *β-ACT8*, which is not responsive to ABA (Saez et al., 2006). Aliquots of the total chromatin input were previously used to provide a quantitative measurement of the DNA input present in each sample, and DNA amounts present in ChIP precipitates were measured using cycle threshold values from RT-qPCR

Figure 4. (continued).

(D) Reduced sensitivity to ABA-mediated inhibition of seed germination in *swi3b-3* and *swi3b-4* mutants compared with Col-*er105*. The percentage of seeds that showed radicle emergence at 96 h after seed stratification is shown. Values are averages ± SD for three independent experiments (n = 200 seeds per experiment). Asterisks are as described for **(B)**.

(E) Reduced sensitivity to ABA-mediated inhibition of early growth in the *swi3b-3* mutant compared with Col-*er105* in medium supplemented with 0.8 μM ABA. The photograph was taken at 18 d after sowing.

(F) Reduced expression of ABA-inducible genes in *swi3b-3* compared with Col-*er105*. Values are expression levels reached in the mutant with respect to Col-*er105* (value 1) as determined by RT-qPCR analyses. Expression of gene markers was analyzed in 7-d-old seedlings grown in medium supplemented with 0.3 μM ABA. Values are averages ± SD for three independent experiments (n = 30 to 40 seedlings per experiment).

(G) The *swi3b-3* phenotype is epistatic to *hab1-1*. The percentage of seeds that showed radicle emergence at 96 h after seed stratification is shown. Values are averages ± SD for three independent experiments (n = 200 seeds per experiment). * $P < 0.01$ (Student's t test) when comparing data from *swi3b-3* and the wild type, or *hab1-1swi3b-3* and *hab1-1*, in the same assay conditions.

(H) Reduced sensitivity to ABA-mediated inhibition of early growth in *swi3b-3* and the *hab1-1swi3b-3* double mutant. Photographs were taken at 7 d (MS) and 11 d (0.5 μM ABA) after sowing. As in **(C)**, plants were removed from growth medium and rearranged on plates for photography.

[See online article for color version of this figure.]

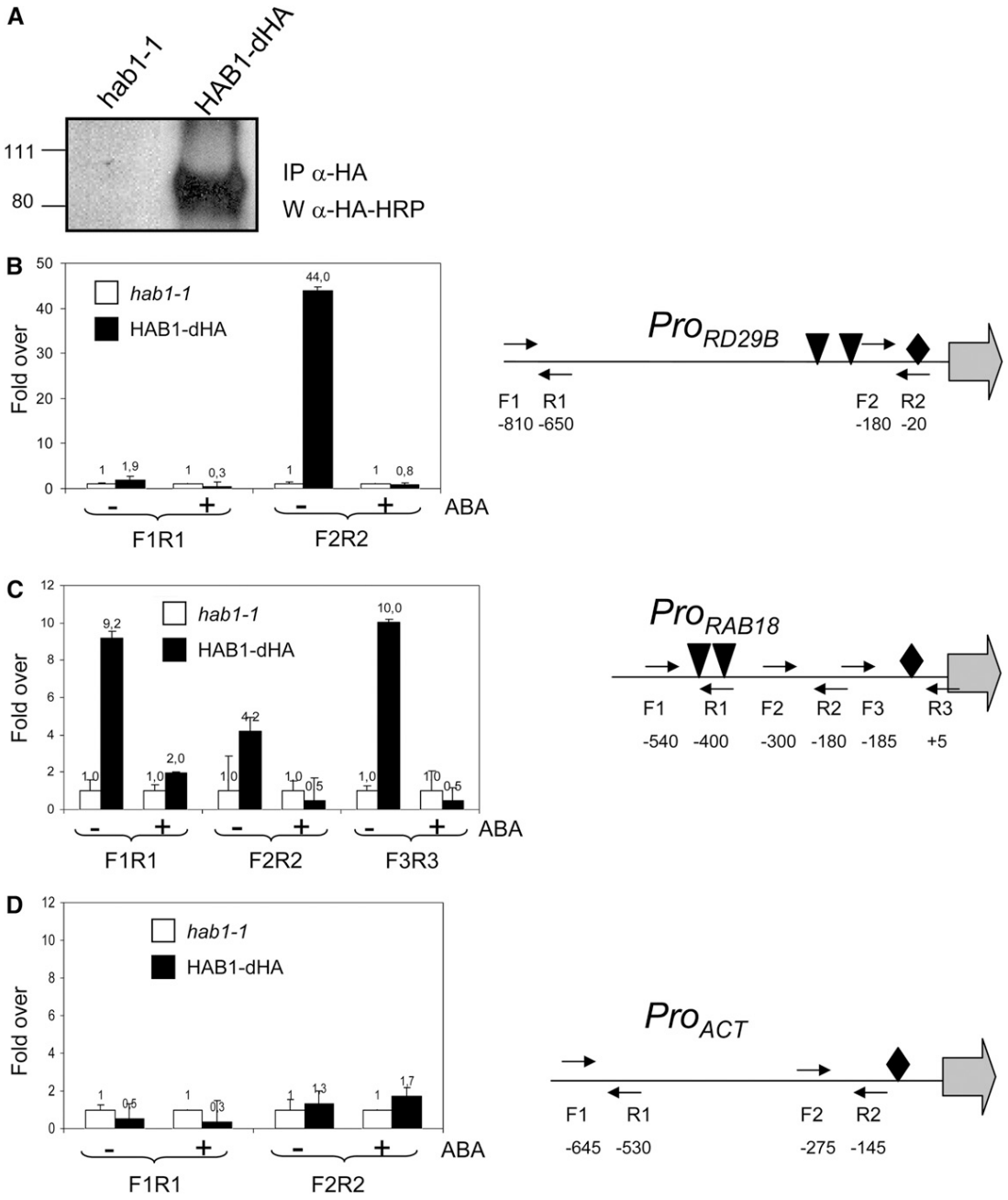

Figure 5. The Presence of HAB1 in the Vicinity of the ABA-Responsive *RD29B* and *RAB18* Promoters Is Abolished by ABA.

(A) Immunoprecipitated samples (IP anti-HA) were subjected to immunoblot analysis (W anti-HA-HRP).
(B) to **(D)** ChIP assays of the *RD29B*, *RAB18*, and *ACT8* promoters in *hab1-1* or *hab1-1::ProHAB1-HAB1-dHA* plants. Genomic DNA fragments that coimmunoprecipitated with HAB1-dHA were analyzed by RT-qPCR using primers of the *RD29B* **(B)**, *RAB18* **(C)**, and *ACT8* **(D)** promoters. Results are presented as ratios of the amount of DNA immunoprecipitated from HAB1-dHA samples to that from the *hab1-1* control (value set to equal 1). The positions of the ABA-responsive elements (triangles) and TATA boxes (diamonds) in the sequences of the different promoters are indicated, as well as the primers used for RT-qPCR analysis. The numbering refers to the ATG translation start codon, and the beginnings of the open reading frames are indicated by arrows.

amplification curves (see Methods). Figures 5B and 5C show that the amounts of *RD29B* and *RAB18* promoter DNAs immunoprecipitated from the HAB1-dHA transgenic plants were over 40- and 10-fold higher, respectively, than that precipitated from *hab1-1* control plants, whereas the amount of *β-ACT8* promoter DNA immunoprecipitated was very similar in both cases (Figure 5D). Interestingly, HAB1 was enriched in those regions of the *RD29B* and *RAB18* promoters that were close to ABA-responsive elements and TATA boxes, and after treatment with 50 μM ABA for 1 h the presence of HAB1 in these regions was abolished (Figures 5B and 5C). *35S-HAB1* lines showed reduced expression of ABA-inducible genes compared with the wild type (Saez et al., 2004); conversely, the *hab1-1* loss-of-function mutant showed twofold higher expression of ABA-inducible genes than the wild type (Saez et al., 2006). These data, together with ChIP results, suggest that HAB1 might repress ABA-induced transcription through direct chromatin interaction and that ABA treatment seems to release such inhibition.

DISCUSSION

Both gain-of-function and loss-of-function phenotypes of the PP2C HAB1 are consistent with a role as a negative regulator of ABA signaling (Leonhardt et al., 2004; Saez et al., 2004). Thus, whereas constitutive expression of *HAB1* (*35S:HAB1*) led to reduced ABA sensitivity in both seeds and vegetative tissues, the recessive *hab1-1* mutant showed ABA-hypersensitive inhibition of seed germination and growth, enhanced ABA-mediated stomatal closure, and enhanced expression of ABA-responsive genes (Leonhardt et al., 2004; Saez et al., 2004, 2006). The ABA-hypersensitive phenotype of *hab1-1* was strongly reinforced when combined with a loss-of-function allele of *ABI1* (Saez et al., 2006). A critical aspect to improve our knowledge on HAB1 function and its role in ABA signaling is the identification of its interacting partners.

Physical Interaction of HAB1 and SWI3B

A two-hybrid assay revealed a strong interaction between the HAB1 catalytic domain and *SWI3B* (Figure 1A). Serial deletions of SWI3B mapped the interacting domain to the N-terminal half of the protein. Thus, both the SWIRM and ZZ zinc finger domains appeared to be required for the interaction, as deletion of either of them abolished the interaction (Figure 1A). The SWIRM (for SWI3-RSC-MOIRA) domain is a small α-helical domain of ~85 amino acid residues found in chromosomal proteins and is predicted to mediate protein–protein interactions in the assembly of chromatin/protein complexes (Aravind and Iyer, 2002; Da et al., 2006). The ZZ zinc finger domain is also likely involved in mediating specific protein–protein interactions with transcriptional adaptors and activators (Ponting et al., 1996). Interestingly, the C-terminal half of SWI3B, which contains both the SANT (for SWI3-ADA2-NCoR-TFIIIB) and Leu zipper domains, was dispensable for the interaction with HAB1 (Figure 1A). However, the mutations found in the *swi3b-3* and *swi3b-4* alleles provide evidence for the importance of the SANT domain for SWI3B function. The equivalent C-terminal half of SWI3C constituted the region that interacted with the ATPase BRAHMA (Hurtado et al., 2006), which also interacted weakly with SWI3B; therefore, it seems likely that such a region plays a similar role in SWI3B. The SANT domain included in this region is structurally related to the Myb DNA binding domain; however, there is no evidence that the SANT domain directly contacts DNA. Instead, SANT domains may directly bind the N-terminal histone tails (Mohrmann and Verrijzer, 2005). Finally, it is suggested that the Leu zipper functions as a homodimerization and heterodimerization domain (Mohrmann and Verrijzer, 2005).

The HAB1 mutant allele G246D ΔNhab1, which had <3% in vitro PP2C activity than the wild type, did not interact with SWI3B. The G246D substitution affects the catalytic center of the PP2C, and according to the crystal structure of human PP2C (Das et al., 1996) such a mutation is expected to disturb the metal-coordinating residues Asp-243 and Gly-244 with concomitant reduction in catalytic activity. An alternative possibility has been postulated by Robert et al. (2006), who suggested that hab1^{Gly246Asp} might show enhanced affinity for its substrate and therefore enhanced dephosphorylating capacity. However, using casein as a substrate, the in vitro PP2C activity of hab1^{Gly246Asp} was severely reduced compared with that of the wild type, as was the case for G246D ΔNhab1. Additionally, the equivalent Gly-180→Asp abi1-1 and Gly-168→Asp abi2-1 mutant proteins did not show enhanced affinity (just the opposite) for their interacting partners, ATHB6/OST1 and SOS2/Prefibrillin, respectively (Himmelbach et al., 2002; Ohta et al., 2003; Yang et al., 2006; Yoshida et al., 2006a). In all of these cases, including the interaction of HAB1 and SWI3B, it appears that a functional catalytic PP2C is required for binding of the different targets.

The interaction of HAB1 and SWI3B was confirmed in planta through BiFC and coimmunoprecipitation assays (Figure 3). HAB1 is localized in both nucleus and cytosol; however, the

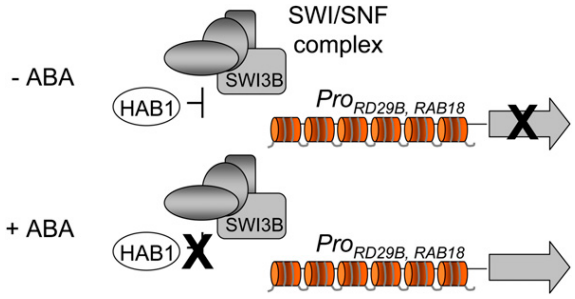

Figure 6. A Model for the Involvement of HAB1, SWI3B, and a Putative Plant SWI/SNF Complex in the Regulation of Plant Transcriptional Response to ABA on the Chromatin Template.

HAB1 is a negative regulator of ABA signaling that interacts with SWI3B, which is a positive regulator of ABA signaling. SWI3B must play a key role as a core subunit of an unidentified SWI/SNF complex, which is predicted to regulate nucleosomal structure in response to ABA. In the absence of ABA, HAB1 is localized in the vicinity of the *RAB18* and *RD29B* promoters and negatively regulates the expression of these genes. ABA inhibits HAB1 and releases its inhibitory effect on a putative SWI/SNF complex. [See online article for color version of this figure.]

BiFC assay clearly identified SWI3B as a nuclear target of HAB1. Interestingly, most of the targets previously identified for clade A PP2Cs were not nuclear proteins (Cherel et al., 2002; Guo et al., 2002; Ohta et al., 2003; Miao et al., 2006; Yang et al., 2006; Yoshida et al., 2006a). However, in the case of ABI1, it is supposed that the interaction with the TF ATHB6 must be nuclear (Himmelbach et al., 2002). Additionally, recent results reveal a nuclear localization signal at the very end of the C-terminal domain of ABI1 that is required for regulating ABA-dependent gene expression (Moes et al., 2008). Inspection of the C-terminal amino acid sequences of HAB1, ABI2, and PP2CA also reveals a similar short region enriched in basic amino acids (see Supplemental Figure 5 online). Additionally, the sequences of HAB1 and ABI2 display a second region that contains two positively charged clusters separated by a short linker region (see Supplemental Figure 5 online). The nuclear interaction of PP2CA, ABI1, and ABI2 with SWI3B found in BiFC assays might be physiologically relevant to regulating ABA signaling. However additional experiments (e.g., ChIP analysis) will be required to confirm the presence of these PP2Cs in plant chromatin and specifically in ABA-regulated promoters. Finally, it is noteworthy that previously described SWI3B-interacting partners connect SWI3B with different components of putative SWI/SNF complexes and, intriguingly, with the RNA and ABA binding protein FCA (Razem et al., 2006). In addition to FCA, six different SWI3B-interacting proteins have been described, namely SWI3A, SWI3C, SWI3D, BRM, SYD, and BSH, which are putative components of SWI/SNF complexes (Sarnowski et al., 2002, 2005; Bezhani et al., 2007). Analysis of the ABA response in mutants affected in these genes will be required for the identification of additional components of SWI/SNF complexes involved in ABA signaling.

Role of HAB1, SWI3B, and a Putative SWI/SNF Complex in ABA Signaling

No SWI/SNF complex has been biochemically purified in plants, although comparative genome analysis indicates that plants encode a remarkably high number of potential components of such a complex (Sarnowski et al., 2005). In yeast, *Drosophila*, and mammals, it is well known that an important subset of highly inducible genes requires a SWI/SNF complex as a transcriptional activator (Mohrmann and Verrijzer, 2005). It has been reported previously that *hab1-1* mutants show twofold higher expression of ABA-responsive genes than wild-type plants (Saez et al., 2006), whereas *35S:HAB1* plants show reduced expression of ABA-inducible genes (Saez et al., 2004); therefore, HAB1 negatively regulates the expression of these genes. HAB1 is localized in both nucleus and cytosol and, therefore, could influence ABA signaling at different steps. ChIP experiments reveal the presence of HAB1 in the vicinity of the ABA-responsive *RAB18* and *RD29B* promoters, and ABA treatment eliminates HAB1 from these regions (Figure 5). These results, taken together with the negative effect of HAB1 on the expression of ABA-inducible genes, strongly suggest a direct regulatory effect of HAB1 on ABA-mediated transcriptional regulation. Thus, the presence of HAB1 in the vicinity of ABA-responsive promoters correlates with the inhibition of their transcription under basal conditions, whereas ABA-mediated removal of HAB1 from these regions appears to be required for full induction of them (Figure 6). In this context, both the HAB1–SWI3B interaction and the impaired upregulation by ABA of *RAB18* and *RD29B* in *swi3b-3* suggest that HAB1 might regulate a putative SWI/SNF complex targeted to some ABA-responsive promoters (Figure 6). The phenotypes described in this work for *swi3b-1* and *swi3b-2* +/− seedlings as well as *swi3b-3* and *swi3b-4* mutants are consistent with SWI3B acting as a positive regulator of ABA signaling. Taking into account the opposed roles of HAB1 and SWI3B in this signaling pathway, it is reasonable to postulate that HAB1 negatively regulates SWI3B function, modulating its role as a positive regulator of ABA signaling. Alternatively, SWI3B might anchor HAB1 to a putative SWI/SNF complex, where the phosphatase activity of HAB1 might dephosphorylate a component required for proper function of the chromatin remodeler. Taking into account that the presence of HAB1 in the vicinity of the ABA-responsive *RD29B* and *RAB18* promoters is abolished by ABA (Figure 5), we speculate that ABA must inhibit HAB1 function, which releases its inhibitory effect on a yet unknown SWI/SNF complex involved in the transcriptional activation of ABA-responsive genes (Figure 6). Finally, it will be an exciting challenge for the future understanding of how the dynamic structure of the chromatin is modulated in response to ABA to regulate gene expression as well as to characterize the cell signaling events that lead to chromatin remodeling.

METHODS

Plant Material

Arabidopsis thaliana (ecotype Col) and tobacco (*Nicotiana benthamiana*) plants were routinely grown under greenhouse conditions in pots containing a 1:3 perlite:soil mixture. For in vitro culture, *Arabidopsis* seeds were surface-sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5%) containing 0.05% Triton X-100 for 10 min, and finally, four washes with sterile distilled water. Stratification of the seeds was conducted during 3 d at 4°C. Afterward, seeds were sown on Murashige and Skoog (MS) plates containing solid medium composed of MS basal salts and 1% sucrose, solidified with 1% agar, and pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled-environment growth chamber at 22°C under a 16-h-light/8-h-dark photoperiod at 80 to 100 $\mu E \cdot m^{-2} \cdot s^{-1}$.

The *swi3b-1* (Koncz_2208) and *swi3b-2* (GABI_302G08) alleles are T-DNA mutants in the Col background. They were kindly provided by G. Rios and have been described previously (Sarnowski et al., 2005). TILLING mutants were obtained through the *Arabidopsis* TILLING project, which performed a high-throughput reverse genetic screening to identify ethyl methanesulfonate–induced mutations in the Col-*er105* background (Till et al., 2003). As a result, two alleles were identified, *swi3b-3* and *swi3b-4*, which showed changes with SIFT score < 0.05 and, therefore, were predicted to be deleterious to the gene product (Ng and Henikoff, 2001). These mutants were backcrossed once with Col-*er105*, and F2 homozygous mutants were genotyped by PCR amplification and DNA sequencing using the primers F1261 and R1560. In the case of *swi3b-3*, a second backcross was done with Col, and F2 *swi3b-3* mutants lacking the *er105* mutation were selected. In order to generate the *hab1-1swi3b-3* double mutant, we transferred pollen of *swi3b-3* (Col background) to the stigmas of emasculated flowers of *hab1-1* (Col background). The resulting F2 individuals were genotyped by PCR for the presence of the double mutant.

Yeast Two-Hybrid Screening

The HAB1 coding sequence was excised from a pSK-HAB1 construct (Rodriguez et al., 1998b) using *Eco*RI-*Sal*I double digestion and subcloned into *Eco*RI-*Sal*I doubly digested pGBT9 to generate an in-frame fusion with the GBD. To generate the HAB1 deletion, the HAB1 sequence encoding the catalytic PP2C region (amino acid residues 179 to 511; ΔNHAB1) was amplified by PCR and blunt-end-cloned into the *Eco*RV site from pBluescript SK+ (Stratagene). The ΔNHAB1 coding sequence was excised with *Eco*RI-*Sal*I and subcloned into pGBT9. The pGBT9-*ΔNHAB1* bait was transformed into the yeast strain AH109 (BD Biosciences). An oligo(dT)-primed cDNA library prepared in plasmid pACT2 using mRNA from an *Arabidopsis* cell suspension was kindly provided by K. Salchert (Nemeth et al., 1998). Yeast host AH109 carrying the pGBT9-*ΔNHAB1* bait was transformed with 100 μg of DNA from the pACT2 cDNA library, then the cells were plated on SCD medium lacking Leu and Trp. Approximately 10^6 clones were obtained, and upon plating in SCD medium lacking Leu, Trp, His, and adenine, 20 clones containing putative interacting preys were selected. Yeast DNA was recovered and electroporated into *Escherichia coli* strain MC1065. pACT2 clones containing putative interacting preys were sequenced and retransformed into yeast strain AH109 carrying either the empty vector pGBT9 or pGBT9-*ΔNHAB1* bait in order to verify true positives.

The PP2CA cDNA was obtained from the ABRC (clone M76G17STM). The PP2CA sequence encoding the catalytic PP2C region (amino acid residues 90 to 399) was amplified using the primers FDNPP2CA and RPP2CA. The PCR product was cloned into the pCR8/GW/TOPO entry vector (Invitrogen), and the ΔNPP2CA-coding sequence was excised with *Eco*RI-*Sal*I and subcloned into pGBT9. The ABI1 and ABI2 cDNAs were kindly provided by Erwin Grill and have been described previously (Meyer et al., 1994; Rodriguez et al., 1998a). The ABI1 sequence encoding the catalytic PP2C region (amino acid residues 122 to 433) was excised using *Eco*RI-*Pst*I double digestion and subcloned into pGBKT7 to generate pGBKT7-*ΔNABI1*. The ABI2 sequence encoding the catalytic PP2C region (amino acid residues 96 to 423; ΔNHAB2) was excised using *Sca*I-*Sal*I double digestion and subcloned into pGBT9 to generate pGBT9-*ΔNABI2*.

Construction of Plasmids

pACT2-SWI3B-C1 was generated from the pACT2-SWI3B full-length cDNA recovered from the two-hybrid screening through *Xho*I digestion and subsequent religation. pACT2-SWI3B-C2, pACT2-SWI3B-N1, pACT2-SWIRM, and pACT2-ZZ were generated through PCR-mediated amplification using the following primer pairs, respectively: FATG and R660, F661 and R1410, FATG and R420, and F400 and R660. Constructs that express fusion proteins between the GAD and SWI3A, SWI3B, SWI3C, or SWI3D in the centromeric vector pPC86 were kindly provided by J.C. Reyes (CABIMER), and they have been described by Hurtado et al. (2006). Protein fusion between the GBD and ΔNHAB1 were generated in the multicopy vector pGBT9 for the yeast two-hybrid screening or the centromeric vector pDBLeu for targeted interaction assays with SWI3-like proteins. The G246D mutation was introduced into the pGBT9-*ΔNHAB1* construct through replacement of a *Bgl*II-*Eco*RV fragment of HAB1 with a PCR-mutagenized version (see below).

Constructs to investigate the subcellular localization of HAB1 and SWI3B were generated in Gateway-compatible vectors. To this end, the coding sequences of HAB1 and SWI3B were PCR-amplified using the following primer pairs, respectively: FBamHI and Rno-stop, and FATG and R1407no-stop. The PCR products were cloned into the pCR8/GW/TOPO entry vector (Invitrogen) and recombined by LR reaction into the pMDC83 destination vector (Curtis and Grossniklaus, 2003).

Constructs to investigate in planta interaction using BiFC assays were made in the pSPYNE-35S and pSPYCE-35S vectors (Walter et al., 2004) as well as the Gateway vector pYFP^{N}43 (kindly provided by A. Ferrando, Universidad de Valencia). The coding sequences of HAB1 and G246D hab1 were excised from pCR8/GW/TOPO constructs using double digestion with *Bam*HI-*Stu*I and subcloned into pSPYCE doubly digested *Bam*HI-*Sma*I. The N-terminal half of SWI3B was excised from a pSK-SWI3B construct using double digestion with *Bam*HI-*Dra*I and subcloned into pSPYNE and pSPYCE doubly digested *Bam*HI-*Sma*I. Constructs in which the basic Leu zipper TF bZIP63 is cloned in pSPYNE-35S and pSPYCE-35S were kindly provided by J. Kudla (University of Münster). The coding sequences of ABI1, ABI2, and PP2CA were PCR-amplified and cloned into pCR8/GW/TOPO and recombined by LR reaction into the pYFP^{N}43 destination vector.

Expression and Purification of MBP-HAB1, MBP-ΔNHAB1, and MBP-G246D ΔNhab1

The coding region of the HAB1 cDNA (Rodriguez et al., 1998b) was PCR-amplified using the primers FSphI and RSphISacI. The PCR product was cloned subsequently into the *Eco*RV site of pBluescript SK (Stratagene), generating pSK-HAB1. Next, an *Eco*RI-*Sal*I DNA fragment was excised from pSK-HAB1 and subcloned into the pMal-c2 vector (New England Biolabs). In order to obtain an N-terminal deletion of HAB1 (ΔNHAB1), a *Hin*dIII DNA fragment encompassing the amino acid residues 116 to 511 was excised from pSK-HAB1 and subcloned into the pMal-c2 vector. HAB1 cDNA was mutagenized by PCR in order to engineer a G246D substitution (Ho et al., 1989). To this end, the following oligonucleotides were used as primers: FPCR1 (5′-TATGATGGTCATGACGGCCATA-AGGTT-3′), in which the codon for Gly-246 (GGA) was changed to Asp (GAC), RATT380, FATTATG, and RPCR2. Once the pMalc2-based constructs were verified by sequencing, expression of recombinant MBP-HAB1, MBP-ΔNHAB1, and MBP-G246D ΔNhab1 was induced with 1 mM isopropylthio-β-galactoside in *E. coli* DH5α cells. The fusion proteins were purified by amylose affinity chromatography according to the manufacturer's instructions (New England Biolabs).

PP2C Activity Assays

Phosphatase activity was measured using ^{33}P-labeled casein as a substrate. Dephosphorylated casein (P-4765; Sigma-Aldrich) was ^{33}P-labeled with bovine heart cAMP-dependent protein kinase (P-5511; Sigma-Aldrich) in a 500-μL reaction volume containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 60 μM cAMP, 50 μM unlabeled ATP, and 0.1 μCi/μL [γ-^{33}P]ATP. The radiolabeled casein was precipitated with 20% trichloroacetic acid, and after two washings with 10% trichloroacetic acid, the casein was dissolved in 200 mM Tris-HCl, pH 7.6. Phosphatase assays were performed in a 50-μL reaction volume containing 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, and ~10,000 cpm of ^{33}P-labeled casein. After incubation for 30 min at 30°C, the reaction was stopped with 100 μL of 20% trichloroacetic acid, samples were centrifuged, and the release of ^{33}Pi in the supernatant was determined by scintillation counting.

Transient Protein Expression in Tobacco

Experiments were performed basically as described by Voinnet et al. (2003). The different binary vectors described above were introduced into *Agrobacterium tumefaciens* C58C1 (pGV2260) (Deblaere et al., 1985) by electroporation, and transformed cells were selected on Luria-Bertani plates supplemented with kanamycin (50 μg/mL). Then, they were grown in liquid Luria-Bertani medium to late exponential phase and cells were harvested by centrifugation and resuspended in 10 mM MES-KOH, pH 5.6, containing 10 mM $MgCl_2$ and 150 μM acetosyringone to an OD_{600} of 1. These cells were mixed with an equal volume of *Agrobacterium* C58C1

(pCH32 35S:p19) expressing the silencing suppressor p19 of *Tomato bushy stunt virus* (Voinnet et al., 2003) so that the final density of *Agrobacterium* solution was ~1. Bacteria were incubated for 3 h at room temperature and then injected into young fully expanded leaves of 4-week-old tobacco plants. Leaves were examined after 3 to 4 d with a Leica TCS-SL confocal microscope and a laser scanning confocal imaging system. Samples for immunoblot and immunoprecipitation assays were harvested, frozen in liquid nitrogen, and stored at −80°C.

Germination and Growth Assays

To measure ABA sensitivity, seeds (~200 seeds per experiment) were plated on solid medium composed of MS basal salts, 1% sucrose, and increasing concentrations of ABA. In order to score seed germination, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined. ABA-resistant growth from *swi3b* +/− heterozygous seedlings (~20 seedlings per experiment) was scored by weighing whole plants after 12 d of the transfer of 5-d-old seedlings grown on 0.5 μM ABA onto MS plates supplemented with 10 μM ABA. Heterozygous individuals from the *swi3b-1* or *swi3b-2* progeny were identified by their hygromycin or sulfadiazine resistance, respectively.

Protein Extraction, Protein Blot Analysis, and Immunoprecipitation

Protein extracts for immunodetection experiments were prepared from either tobacco leaves infiltrated with *Agrobacterium* or transgenic lines from *Arabidopsis*. Plant material (~200 mg) for protein gel blot analysis was directly extracted in 2× Laemmli buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol, and 0.001% bromphenol blue), and proteins were run on a 10% SDS-PAGE gel and analyzed by immunoblotting. Plant material (~1 g) for immunoprecipitation experiments was extracted in 3 volumes of PBS supplemented with 1 mM EDTA, 0.05% Triton X-100, and protease inhibitor cocktail (Roche). Protein concentration in each lysate was adjusted to the same value, and equal volumes of lysates (1 mL) were incubated with 1 μg/mL anti-HA high-affinity rat monoclonal antibody (clone 3F10; Roche) for 4 h at 4°C. After incubation, 20 μL of protein G–agarose beads (Roche) was added to precipitate the antigen/antibody complex. The protein G–agarose beads were collected after 1 h of incubation at 4°C by centrifugation and washed three times with extraction buffer. The antigen/antibody complex was eluted by boiling in Laemmli buffer and run on a 10% SDS-PAGE gel. Proteins immunoprecipitated with anti-HA antibodies were transferred onto Immobilon-P membranes (Millipore) and probed with either anti-HA-peroxidase or anti-c-myc-peroxidase conjugate (Roche), and detection was performed using the ECL advance protein gel blotting detection kit (GE Healthcare). The imaging of the chemiluminiscent signal was achieved using a highly efficient cooled CCD camera system (LAS-3000 luminiscent image analyzer from Fuji Photo Film). The signal intensities of the digitalized images were quantified using Image-Gauge version 4.0 software (Fuji Photo Film) according to the manufacturer's conditions. Immunodetection of GFP fusion proteins was performed with an anti-GFP monoclonal antibody (clone JL-8; Clontech) as primary antibody and ECL anti-mouse peroxidase (GE Healthcare) as secondary antibody. A rabbit antibody against peptide comprising amino acids 3 to 17 of GFP (anti-GFP^N) was employed to detect YFP^N fusion proteins (G1544; Sigma-Aldrich).

RNA Analyses

Plants were grown on MS plates supplemented with 1% sucrose either in the absence or presence of 0.3 μM ABA. After 7 d, ~30 to 40 seedlings were collected and frozen in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy plant mini kit, and 1 μg of the RNA solution obtained was reverse-transcribed using 0.1 μg of oligo$(dT)_{15}$ primer and Moloney murine leukemia virus reverse transcriptase (Roche), to finally obtain a 40-μL cDNA solution. RT-qPCR amplifications and measurements were performed using an ABI PRISM 7000 sequence detection system (Perkin-Elmer Applied Biosystems). RT-qPCR amplifications were monitored using the Eva-Green fluorescent stain (Biotium). Relative quantification of gene expression data was performed using the $2^{-\Delta\Delta C_T}$ (or comparative C_T) method (Livak and Schmittgen, 2001). Expression levels were normalized using the C_T values obtained for the *β-actin8* gene. The presence of a single PCR product was further verified by dissociation analysis in all amplifications. All quantifications were made in triplicate on RNA samples obtained from three independent experiments. The sequences of the primers used for PCR amplifications are indicated at Supplemental Table 1 online.

Generation of Epitope HA-Tagged HAB1 Transgenic Lines

The pBluescriptSK-*ProHAB1:HAB1* construct was described by Saez et al. (2004). Two copies of the HA epitope sequence encoding YPYDVP-DYA were cloned at the C-terminal sequence of *HAB1* cDNA in the construct mentioned above. The complete expression cassette comprising the *HAB1* promoter, the double HA epitope–tagged *HAB1* coding sequence, and the *NOS* terminator was subcloned into *Sac*I-*Sal*I doubly digested pCAMBIA 1300 (hygromycin-resistant). The resulting construct was named pCAMBIA1300-*ProHAB1:HAB1-dHA* and used to transform *hab1-1* (kanamycin-resistant) plants as described by Saez et al. (2004). Transgenic plants were screened in vitro on MS medium (M5524; Sigma-Aldrich) with 20 mg/L hygromycin B (H9773; Sigma-Aldrich).

Biochemical Fractionation of Epitope HA-Tagged HAB1

This protocol is based on fractionation techniques described by Bowler et al. (2004), Poveda et al. (2004), and Cho et al. (2006). Rosette leaves from 3- to 4-week-old plants were mock-treated or treated with 50 μM ABA for 1 h, harvested, and frozen in liquid nitrogen. Next, plant material was ground in lysis buffer (20 mM Tris-HCl, pH 7.4, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM $MgCl_2$, 250 mM sucrose–containing protease inhibitor cocktail [Roche], and 1 mM phenylmethylsulfonyl fluoride [PMSF]). The lysate was filtered through four layers of Miracloth and centrifuged at 1000*g* for 10 min at 4°C to pellet the nuclei. The soluble cytosolic fraction was removed, and the pellet was washed in nuclei resuspension buffer, 20 mM Tris-HCl, 25% glycerol, 2.5 mM $MgCl_2$, and 0.5% Triton X-100. After centrifugation at 1000*g* for 30 s at 4°C, a nuclear pellet was obtained, which was resuspended in 5 volumes of medium salt buffer (Bowler et al., 2004), 20 mM Tris-HCl, 0.4 M NaCl, 1 mM EDTA, 5% glycerol, 1 mM 2-mercaptoethanol, 0.1% Triton X-100, 0.5 mM PMSF, and protease inhibitor cocktail (Roche) and then frozen and thawed. After incubation with gentle mixing for 15 min at 4°C, the nuclear insoluble fraction, containing the major nuclear protein histones, was precipitated by centrifugation at 10,000*g* for 10 min, whereas the supernatant contained the nuclear soluble fraction. Detection of HAB1 was performed using anti-HA peroxidase conjugate (Roche). The purity of the different fractions was demonstrated using rabbit antibodies against histone H3 (Abcam) and ribulose-1,5-bisphosphate carboxylase.

ChIP

The ChIP protocol described here is a variation of the previously published protocols from Johnson et al. (2002) and Pascual-Ahuir et al. (2006). A transgenic line of Arabidopsis expressing a double HA epitope–tagged HAB1 in a *hab1-1* background was used as starting plant material. In parallel, plant material from the *hab1-1* mutant was used as a control for the experiment. Rosette leaves from 3- to 4-week-old plants were

mock-treated or treated with 50 μM ABA for 1 h and then harvested and immersed in buffer A (0.4 M sucrose, 10 mM Tris, pH 8, 1 mM EDTA, 1 mM PMSF, and 1% formaldehyde) under vacuum for 10 min. Gly was added to a final concentration of 0.1 M, and incubation was continued for an additional 5 min under vacuum. Next, the plant material was washed with TBS (20 mM Tris-HCl, pH 8, and 150 mM NaCl) and frozen in liquid nitrogen. Cross-linked material (~1 g) was ground with a mortar with pestle, after which it was resuspended in 1 mL of ice-cold lysis buffer (50 mM HEPES/KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, and 1 mM PMSF) and transferred to a 2-mL screw-cap vial. Approximately 0.5 mL of zirconia/silica beads was added, and plant material was disrupted at 4°C for 10 min in the Mini Bead Beater 8 (Biospec Products; maximal speed, three rounds of 1 min). The lysate was collected into a 1.5-mL microtube and centrifuged for 1 min at 4°C. The pellet was collected and washed once in 1 mL of ice-cold lysis buffer. Next, the pellet was resuspended in 0.5 mL of cold lysis buffer and sonicated three times for 20 s (Branson Sonifier; output, 50%; needle, 5). Sonication resulted in the fragmentation of the chromatin into soluble pieces in the range of 300 to 500 bp. After centrifugation for 30 min at 4°C, the supernatant containing the soluble chromatin fragments (chromatin input) was transferred to 1.5-mL microtubes and stored at −80°C.

To immunoprecipitate HAB1-dHA cross-linked to chromatin fragments, samples were incubated with 10 μg/mL anti-HA high-affinity rat monoclonal antibody (clone 3F10; Roche) for 30 min on a roller at room temperature. In the meantime, protein G–agarose beads were washed twice with lysis buffer, 25 μL was added to each sample, and the incubation was continued for 60 min. The agarose beads were recovered by centrifugation and then washed with 1 mL of each of the following buffers: 2× lysis buffer, 2× lysis buffer and 0.5 M NaCl, 1× buffer B (10 mM Tris-HCl, pH 8, 0.25 M LiCl, 1 mM EDTA, 0.5% Nonidet P-40, and 0.5% deoxycholate), and 1× TE (10 mM Tris-HCl, pH 8, and 1 mM EDTA). The immunocomplexes were eluted from the beads by incubation for 10 min at 65°C in 250 μL of buffer containing 50 mM Tris-HCl, pH 8, 10 mM EDTA, and 1% SDS. After centrifugation, the supernatant was transferred to a microtube containing 250 μL of TE buffer and 20 μg of Pronase (Roche), and the samples were incubated for 1 h at 42°C followed by 5 h at 65°C to reverse formaldehyde-induced cross-links. In addition to the immunoprecipitated samples, aliquots (50 μL) of the total chromatin input that were not subjected to immunoprecipitation were also treated with Pronase and de-cross-linked to provide a quantitative measurement of the DNA input present in each sample. Finally, genomic DNA fragments were purified by the addition of 50 μL of 4 M LiCl and extraction with 300 μL of phenol:chloroform:isoamyl alcohol and ethanol precipitation (adding 20 μg of glycogen as carrier). DNA pellets were washed with 70% ethanol, dissolved in 100 μL of TE buffer, and stored at −20°C. RT-qPCR was used to determine the amounts of genomic DNA immunoprecipitated in the ChIP experiment. The sequences of the primers used for PCR amplifications are indicated at Supplemental Table 1 online.

Accession Numbers

The Arabidopsis Genome Initiative locus identifiers for *HAB1* and *SWI3B* are *At1g72770* and *At2g33610*, respectively. *RD29B*, *RAB18*, *KIN1*, *RD22*, *RD29A*, and *P5CS1* correspond to *At5g52300*, *At5g66400*, *At5g15960*, *At5g25610*, *At5g52310*, and *At2g39800*, respectively.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The N-Terminal Region of HAB1 Has Transcriptional Activation Function in Yeast When Fused to the Gal4 DNA Binding Domain.

Supplemental Figure 2. Treatment with 50 μM ABA for Different Periods of Time (5 min to 1 h) Does Not Change the Subcellular Localization of GFP-HAB1.

Supplemental Figure 3. Detached Leaves Water Loss Assays Do Not Show Significant Differences between Wild-Type and *swi3b-1* and *swi3b-2* +/− Plants.

Supplemental Figure 4. Reduced Vegetative and Reproductive Growth of the *swi3b-3* Mutant.

Supplemental Figure 5. Putative Nuclear Localization Signals in HAB1, PP2CA, and ABI2.

Supplemental Table 1. Primers Used for PCR Amplifications.

Supplemental Data Set 1. Text File of the Alignment Corresponding to Supplemental Figure 5.

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